Short Communication

Disulfiram Metabolism in Isolated Mesophyll Cells and Inhibition of Photosynthesis and Cyanide-Resistant Respiration

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ABSTRACT
Tetraethylthiuram disulfide (disulfiram) stimulated medium acidification when added at a concentration of 0.4 millimolar to illuminated or nonilluminated suspensions of Asparagus sprengeri Regel mesophyll cells. Similar concentrations inhibited photosynthesis and cyanide-resistant respiration. The reduction product of disulfiram, diethylidithiocarbamic acid, accumulated in concentrations sufficient to account for the observed acidification.

Disulfiram (tetraethylthiuram disulfide) is a potent inhibitor of cyanide-resistant respiration in isolated mitochondria (7) or intact mesophyll cells of Asparagus sprengeri Regel (17). In addition, disulfiram and other thiuram disulfides inhibit photosynthesis (13, 14). The mechanisms by which disulfiram inhibits these processes are not understood. Inhibition in both cases may result from a reaction between the disulfide component of disulfiram and enzyme thiyl groups essential for the cyanide-resistant pathway or photosynthesis (7, 14, 16). Another possible mechanism of inhibition involves chelation by disulfiram of transition metals located in enzyme systems (14). Inhibition may also be mediated by disulfiram's ability to function as a free radical scavenger and react with fatty acid perox radicals (18). In the present paper, the ability of isolated Asparagus mesophyll cells to rapidly reduce disulfiram to DIECA is demonstrated. The possible involvement of DIECA production in inhibition of either process is investigated.

MATERIALS AND METHODS
Asparagus sprengeri Regel was grown and mesophyll cells isolated using previously described methods (3, 4). Chl content of cell suspensions was determined by the method of Arnon (2) and cell numbers measured with a hemocytometer. A mean value of 36 ± 20 (sd) µg Chl/10⁶ cells was obtained.

Rates of acidification in aerated and stirred cell suspensions maintained at 30°C in 10 ml unbuffered salt solutions, containing 5 mM KCl, 5 mM NaCl, and 0.2 mM CaCl₂, were determined with a recording pH meter (3). Rates were calculated as nmol H⁺/10⁶ cells min or mmol H⁺/mg Chl min.

DIECA in the suspending medium was determined by measuring the absorbance of a copper (II) DIECA complex at 433 nm (1).

Rates of O₂ production in the light or O₂ consumption in the dark were determined with a calibrated O₂ electrode (YSI 4004) using cells suspended in 7.5 ml of a 30°C buffered medium contained within a closed system (3). A concentration of NaHCO₃ saturating for photosynthesis (4) was added to the cell suspension immediately after illumination with a 300-w reflector lamp (Sylvania) began. Irradiance at the surface of the vessel was 1200 × 10⁻³ w cm⁻². Rates were corrected for consumption by the O₂ electrode and expressed as µmol O₂/mg Chl h or µmol O₂/10⁶ cells h.

Stock solutions of DIECA and disulfiram were made using 80% (v/v) ethanol and volumes of KCN, ethanol, DIECA, or disulfiram were dispensed with Hamilton syringes to give the concentrations indicated.

RESULTS

Measurements of net rates of H⁺ efflux were initiated by the addition of 9 to 12 × 10⁶ cells suspended in 3 ml unbuffered salt solution to 7 ml of similar medium. The resulting 10 ml cell suspension was stirred and aerated. The initial value for the net rate of H⁺ efflux varied between 0.3 and 1.8 nmol H⁺/10⁶ cells min and the mean value expressed on a Chl basis was 31.6 nmol H⁺/mg Chl min. Disulfiram stimulated acidification with no apparent lag (Fig. 1). In the light, 0.4 mM disulfiram stimulated acidification to a mean value of 18 nmol H⁺/10⁶ cells min and the overall decline in pHe was equivalent to 396 ± 83 (sd) nmol H⁺. The molar ratio of H⁺ appearing in the medium to disulfiram added was 0.1. In the dark, 0.4 mM disulfiram stimulated the acidification rate to 8.6 nmol H⁺/10⁶ cells min and the pHe decrease was equivalent to 210 ± 64 (sd) nmol H⁺ (Fig. 1). The rate or extent of acidification was not significantly reduced until the disulfiram concentration was decreased to 10 µM. When the normal rate of H⁺ efflux was eliminated with 1 µg/ml oligomycin, acidification in response to disulfiram was still observed. Control experiments showed that the disulfiram solvent ethanol did not stimulate acidification and in the absence of cells neither did disulfiram. The Na⁺ salt of DIECA did not stimulate acidification, and concentrations greater than 40 µM resulted in alkalization of the cell suspension medium.

DIECA in the 10-ml suspension medium was determined after acidification, in response to 0.4 mM disulfiram, was complete and cells were removed by centrifugation. With illumination, a mean value of 1,010 nmol DIECA were produced; without illumination, 960 nmol of DIECA were obtained. These values indicate an approximate 0.1 mM DIECA concentration in the 10-ml suspension medium. The molar ratio of DIECA production to disulfiram employed was 0.25. The ratio of H⁺ to DIECA

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1 Supported by a grant from the Natural Sciences and Engineering Research Council of Canada (NSERC).
2 Abbreviations: DIECA, diethylidithiocarbamic acid; DMO, 5,5-dimethylxalazolidine-2,4-dione; SHAM, salicyldihydroxamic acid; CCCP, carbonyl cyanide m-chlorophenyl-hydrazone.
DISULFIRAM AND MESOPHYLL CELLS

Fig. 1. Stimulation of medium acidification by disulfiram. Recordings of pH change with cells suspended in unbuffered 5 mM KCl, 5 mM NaCl, and 0.2 mM CaCl₂. Recordings obtained in the dark (D) or with illumination (L). Rates of acidification are expressed as nmol H⁺/10⁶ cells-min.

generated in response to disulfiram was 0.40 in the light and 0.21 in the dark. DIECA was not detected when disulfiram was added to aerated and stirred suspension medium lacking cells.

Photosynthetic O₂ production was measured using saturating light and bicarbonate concentrations with 3 to 4 × 10⁶ cells suspended in 7.5 ml of 50 mM Hepes buffer adjusted to pH 7.5 with KOH. The mean rate obtained was 32 ± 6.4 (SD) μmol O₂/mg Chl-h or 1.10 ± 0.22 (SD) μmol O₂/10⁶ cells-h. Addition of 50 μM disulfiram resulted in complete inhibition of O₂ evolution within 2 min (Fig. 2), and 10 μM disulfiram inhibited evolution by approximately 50%. Similar results were obtained with cells suspended in 50 mM K-phosphate (pH 6.2) (Fig. 2). The concentration of ethanol used in these studies had little or no effect on photosynthesis indicating that inhibition was due to disulfiram, not its solvent (Fig. 2). The Na⁺ salt of DIECA at a 0.4 mM concentration had no significant effect on photosynthetic oxygen production.

Fig. 2. Inhibition of photosynthesis by disulfiram. Recordings of O₂ evolution were obtained with cells suspended in 0.05 Hepes buffer (pH 7.2) (A and C) or 0.05 mM phosphate buffer (pH 6.2) (B). The final concentration of disulfiram was 50 μM and the final concentration of ethanol 0.8% (v/v). Rates of photosynthesis are expressed as μmol O₂/10⁶ cells-h.

Respiration rates were measured with nonilluminated cells suspended in either 50 mM Hepes buffer (pH 7.5) or 50 mM K-phosphate (pH 6.2). Rates were linear with time and varied between 0.12 and 0.22 μmol O₂/10⁶ cells-h; the mean rate when expressed on Chl basis was 1.4 μmol O₂/mg Chl-h. Sequential increments in the DIECA sodium salt concentrations up to 0.2

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Fig. 3. Inhibition of cyanide-resistant respiration by disulfiram. All recordings were obtained using 0.05 mM Hepes buffer (pH 7.2). Trace A indicates changes in O₂ concentration in the absence of cells. Additions of KCN, DIECA, and disulfiram are indicated. Rates of O₂ consumption after each addition are expressed as μmol O₂/10⁶ cells-h.
mm resulted in 24% and 28% mean inhibition of the initial rates in the Heps and phosphate buffers, respectively. Subsequent addition of 0.2 mM disulfiram resulted in 58% and 65% mean inhibition of the remaining respiration, indicating that disulfiram is a more potent inhibition than DIECA (Fig. 3). Addition of KCN concentrations up to 100 μM demonstrated that respiration was resistant to cyanide and in some cases respiration was stimulated. The cyanide-resistant respiration was inhibited by a mean value of 14% by 0.2 mM DIECA. Subsequent addition of 0.2 mM disulfiram, however, reduced rates by approximately 50% (Fig. 3).

**DISCUSSION**

Rapid inhibition of photosyntheses and cyanide-resistant respiration by disulfiram indicate that it passes readily into the cell (Figs. 2 and 3). A previous report concluded that, while disulfiram was a potent inhibitor of the alternative pathway in isolated mitochondria, it was without effect on intact tissues or cells (7). However, rapid entry is expected with a nonpolar compound having four ethyl groups when stirring reduces diffusion distances to a minimum. The reduction of disulfiram to DIECA will result in the generation of a free acid group (pK 3.9) which will be largely dissociated at cytoplasmic pH values. Accumulation of DIECA in the unbuffered suspension medium indicates that acidification of this medium (Fig. 1) results from equilibration of undissociated uncharged DIECA across the plasma membrane and with the internal and external pools of the corresponding anions. The molar excess of DIECA over H⁺ generated in the suspension medium indicates that measurements of net H⁺ efflux may be reduced by H⁺ reflux.

Reduction of disulfiram to DIECA could involve a reaction with protein sulfhydryl groups resulting in the production of a protein-mixed disulfide complex and one molecule of DIECA (7, 16). Alternatively, reduction may be catalyzed by an enzyme system such as NADP-linked glutathione reductase or thoredoxin reductase which reduce disulfide compounds (9). In this case, one disulfiram molecule would generate two DIECA molecules. Photosynthetically driven reduction of disulfiram may explain the faster acidification rate in the light (Fig. 1), although the process was not inhibited by DCMU.

The response to disulfiram is strikingly similar to the response of *Asparagus* cells to the addition of lipophilic carboxyester compounds (5). In both cases, rapid metabolism generates an acidic compound, acidification of the medium occurs, photosynthesis is inhibited, and addition of the metabolic products does not inhibit photosynthesis. Inhibition of photosynthesis may arise through an intracellular acidification process which reduces the intracellular pH. Whereas a stromal pH of 7.0 inhibits CO₂ fixation, optimum activity is found at pH 8.0 (19). Acidification could result from the reduction of disulfiram within the stroma or from entry and dissociation of weak acids generated in the cytosol as a result of protonation at a reduced cytosolic pH (6). This interpretation is supported by preliminary experiments in which addition of 10 mM NH₄Cl resulted in a rapid 37% recovery of disulfiram-inhibited O₂ evolution. Similar experiments with concentrations of CCCP sufficient to uncouple photophosphorylation (8) did not result in a renewal of O₂ evolution. These data suggest that the effect of NH₄Cl is not due to an uncoupling of photophosphorylation but are consistent with a reversal of intracellular acidification when membrane-permanent NH₄⁺ is protonated. In addition, the similarity in the responses of *Asparagus* cells to disulfiram and lipophilic carboxyesters which lack a disulfide group (5) suggests that inhibition of photosynthesis may not involve an interaction between functional thiol and disulfide groups. This interpretation contrasts with a previous paper which demonstrated disulfiram reduction to DIECA, but which did not consider intracellular acidification as a possible mechanism by which photosynthesis is inhibited (14).

Most work on cyanide-resistant respiration has focused on tissue slices or mitochondria from fruits or storage organs (12). Two recent reports, however, demonstrate the presence of cyanide-resistant SHAM-sensitive respiration in leaf tissue (11) and cyanide-resistant disulfiram-sensitive respiration in isolated *Asparagus* mesophyll cells (17). Lipophilic carboxyesters had no influence on cyanide-resistant respiration suggesting that disulfiram inhibition does not involve intracellular acidification. The generation of 0.1 mM DIECA concentrations on the addition of 0.4 mM disulfiram indicates that inhibition may involve DIECA. However, disulfiram was a far more potent inhibitor of cyanide-resistant respiration than an equal concentration of DIECA (Fig. 3). The small inhibition observed on addition of DIECA may in fact be due to disulfiram if a reversible redox reaction produces an equilibrium ratio of disulfiram and DIECA (10). The evidence suggests that inhibition does not depend on production of DIECA, and that disulfiram is the inhibitory species. A similar conclusion was reached when disulfiram was used to inhibit cyanide-resistant respiration in isolated mitochondria (7). The residual respiration (Fig. 3) may represent lipoxygenase activity which is insensitive to cyanide and disulfiram (15).

**LITERATURE CITED**

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