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Biochemistry

Reductive detoxification as a mechanism of fungal resistance to singlet oxygen-generating photosensitizers

(cercosporin/rose bengal/redox potential/fluorescence microscopy/cyclic voltammetry)

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ABSTRACT **Fungi that are resistant or sensitive to the singlet oxygen-generating toxin cercosporin were assayed for their ability to detoxify it by reduction. Cercosporin reduction was assayed microscopically by using bandpass filters to differentiate between fluorescence emission from cercosporin and reduced cercosporin. Hyphae of the resistant *Cercospora* and *Alternaria* species emitted a green fluorescence, indicative of reduced cercosporin. Hyphae of nonviable cultures and of cercosporin-sensitive fungi did not reduce cercosporin. Sensitive fungi occasionally reduced cercosporin when incubated with reducing agents that protect against cercosporin toxicity. Cercosporin could not be efficiently photoreduced in the absence of the fungus. *Cercospora* species were also resistant to eosin Y but were sensitive to rose bengal. Microscopic observation demonstrated that *Cercospora* species were not capable of reducing rose bengal but were capable of reducing eosin Y. These observations were supported by *in vitro* electrochemical measurements that revealed the following order with respect to ease of reduction: cercosporin >> eosin Y > rose bengal. The formal redox potential ($E^{\circ\prime}$) of cercosporin at pH 7.5 was found to be −0.14 V vs. the normal hydrogen electrode. We conclude that *Cercospora* species protect themselves against cercosporin by the reduction and detoxification of the toxin molecule.**

Cercosporin is a lipid-soluble perylenequinone toxin that is produced by fungi in the genus *Cercospora* and plays an important role in their ability to parasitize plants (1, 2). Cercosporin causes peroxidation of membrane fatty acids, leading to the rupture of the plant plasma membrane and cell death (3, 4). All plants and bacteria that have been tested are sensitive to cercosporin, as are mice and many fungi (1, 5, 6). The only known resistant organisms are *Cercospora* species and some related fungi, of which several produce similar toxins. Thus, we are interested in defining the cellular basis of toxin resistance.

Cercosporin belongs to a class of molecules called photosensitizers, which are characterized by the ability to be activated by light and to react with oxygen to produce toxic active oxygen species (7). Photosensitizers damage nuclear, membrane, and cytoplasmic components of cells, the precise target being determined by the cellular localization of the photosensitizer molecule (8). Photoactivated cercosporin can react with oxygen both through a reducing agent (type I) and by direct energy transfer (type II) to produce, respectively, superoxide ($O\cdot_2^-$) and singlet oxygen ($^1O_2$) (9–11). Of the two, $^1O_2$ appears to be the dominant species produced by cercosporin both *in vitro* and *in vivo*.

Photosensitizers are a large and ubiquitous group of compounds. They cause diseases of animals and humans, protect plants against predators and pathogens, are involved in photomovement in protozoans, and are used as pesticides and pharmaceuticals (7, 12–14). Notwithstanding the importance of these compounds, little is known about organismal defenses against them. Although plants are major producers of photosensitizers (15, 16), mechanisms of protection against autotoxicity have not been documented. Light-avoidance responses such as photophobic responses, feeding within a rolled leaf, or secretion of protective webs have been documented in protozoans and insects (17, 18). Some insects contain pigments that block light transmission or are able to metabolize the compounds (18). When exposed to bright light, the protozoan *Blepharisma* irreversibly oxidizes its endogenous photosensitizer blepharismin to an inactive form and then extrudes it (17). Molecules that quench or block the formation of active oxygen species are also known, the most efficient of which are carotenoids, which quench both $^1O_2$ and the activated triplet state of photosensitizers (19).

Resistance of *Cercospora* species to cercosporin poses a unique problem, since the fungus requires an active molecule for infection of host plants. Toxin-deficient mutants of the fungus are nonpathogenic (2), and high light intensities are required for normal symptom development (1). Cercosporin accumulates to as high as 1 mM in actively growing, light-incubated cultures with no measurable decrease in fungal growth. Thus, *Cercospora* species appear to have evolved a resistance mechanism that allows them to tolerate high concentrations of active cercosporin without a major expenditure of energy.

Here we present evidence that *Cercospora* species protect themselves against cercosporin by a reversible reduction and detoxification of the toxin molecule. Further, we suggest that photosensitizer reduction may be a generalized mechanism of resistance for these organisms to other $^1O_2$-generating photosensitizers. We used fluorescence microscopy with specific bandpass filters to differentiate fluorescence from cercosporin and reduced cercosporin. Presence of cercosporin was indicated by red fluorescence visualized with a 575- to 635-nm filter. Green fluorescence visualized with a 515- to 545-nm filter was interpreted as indicative of reduced cercosporin.

MATERIALS AND METHODS

Fungal Strains and Culture Conditions. The strains used were *Cercospora nicotianae* (ATCC 18366), *Cercospora kikuchii* PR (provided by J. B. Sinclair, University of Illinois), *Neurospora crassa* ORS-6a (Fungal Genetics Stock Center, University of Kansas), *Aspergillus flavus* (ATCC 60045), and *Alternaria alternata* A5 (provided by H. Spurr, United States Department of Agriculture, Oxford, NC). For

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Abbreviations: $^1O_2$, singlet oxygen; GSH, reduced glutathione; PDA, potato dextrose agar; PDB, potato dextrose broth; CV, cyclic voltammetry.

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microscopic analysis, fungi were grown in shake culture in potato dextrose broth (PDB; Difco) at 25°C in the dark; under these conditions, *Cercospora* species do not synthesize cercosporin. *Cercospora* species were grown for 24–40 hr, *Alt. alternata* and *Asp. flavus* for 24–28 hr, and *N. crassa* for 16–24 hr. Cercosporin, rose bengal, and eosin Y (all at 10 μM) were added 16–18 hr before cultures were viewed under the microscope. Reduced glutathione (GSH), ascorbate, and cysteine were either added (20 mM) to cultures along with cercosporin or provided as 50 mM solutions in PDB that were mixed (1:1) with the cultures immediately before viewing under the microscope. For observation of cercosporin-producing cultures, *C. kikuchii* was grown on potato dextrose agar (PDA; Difco) on microscope slides for 48–72 hr under a 16-hr light [14 $\mu E \cdot m^{-2} \cdot sec^{-1}$ (1 einstein, E = 1 mol of photons)]/8-hr dark cycle. To assay for possible cercosporin reduction by nonviable hyphae, cultures grown on microscope slides were killed by one of three methods; exposure to chloroform vapor or to steam for 30 min or exposure to a germicidal UV lamp for 60 min.

Microscopic Analysis. The 450- to 490-nm excitation filter allowed for excitation of both cercosporin and reduced cercosporin at their absorption maxima (Fig. 1*A*). The 515- to 545-nm bandpass filter ("530" filter) allowed observation only of fluorescence emitted by reduced cercosporin (Fig. 1*B*). The 575- to 635-nm bandpass filter ("605" filter) allowed observation primarily of cercosporin fluorescence, but the filter bandpass overlaps the fluorescence emission spectrum of reduced cercosporin (Fig. 1*B*). Thus, with the 605 filter, fluorescence from reduced cercosporin was distinguished from that of cercosporin by color, yellow vs. red, respectively. Observation of eosin Y treatments utilized the same excitation filter and a barrier filter that transmitted all wavelengths above 520 nm. Rose bengal fluorescence was visualized with a 546-nm excitation filter and a 590-nm barrier filter.

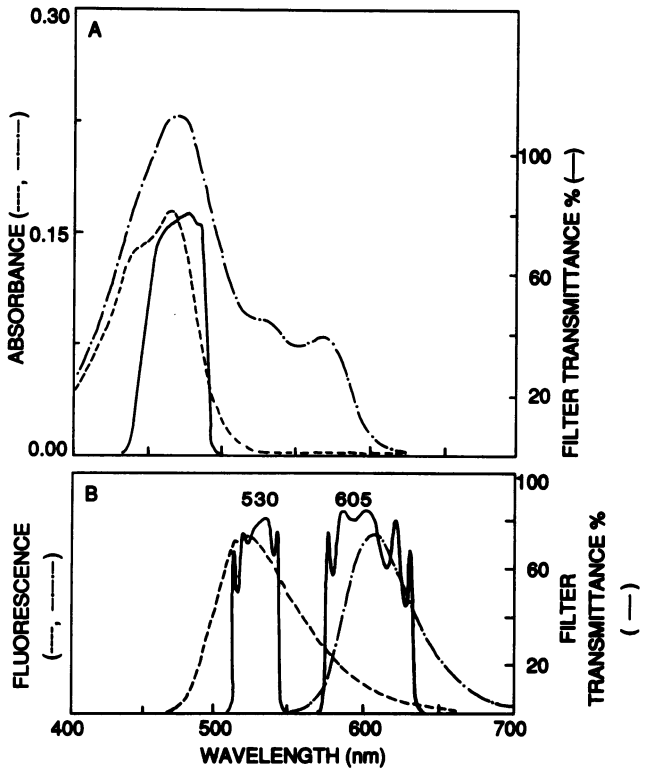

FIG. 1. Excitation and bandpass filters used for detection of cercosporin and reduced cercosporin. (*A*) Excitation filter transmittance (—) and absorption spectra of cercosporin (– · –) and dithionite-reduced cercosporin (- - -), both at 10 μM in 10% acetone. (*B*) Filter transmission of the 530 and 605 bandpass filters (—) and fluorescence emission spectra of cercosporin (– · –) and dithionite-reduced cercosporin (- - -). Fluorescence spectra were normalized to the same peak intensity.

Photosensitizer Resistance. Phototoxicity of rose bengal and eosin Y to *C. kikuchii, N. crassa,* and *Asp. flavus* was compared with that of cercosporin by assaying radial growth on PDA. Each compound was tested at 10 μM, the solubility limit for cercosporin. Test and control plates for cercosporin treatments contained 0.5% acetone. Cultures were incubated at 25°C under constant fluorescent light (75 $\mu E \cdot m^{-2} \cdot sec^{-1}$). Radial growth was measured at 16 hr (*N. crassa*) or 4 days (*C. kikuchii* and *Asp. flavus*).

Photoreduction. Photoreduction of cercosporin was tested on 10 μM solutions in water or PDB containing 10% acetone. Cysteine, ascorbate, and GSH were added at 20 mM. Solutions were sparged with prepurified nitrogen (<5 ppm oxygen) for 12 min. For the last 2 min, the cuvette was exposed to light from a photoflood lamp. Control cuvettes either were made anaerobic but kept dark or were not sparged with nitrogen. Photoreduction was monitored both by a decrease in absorbance at 560 nm and by an increase in fluorescence at 522 nm and was compared with the values for a solution of cercosporin reduced with dithionite (4 mg/ml).

Electrochemistry. Cyclic voltammetry (CV) was performed with a Cypress Systems CYSY-2 potentiostat and a glass cell (20). For cercosporin, indium–tin oxide electrodes (Donnelly, Holland, MI) were cleaned (20) and equilibrated overnight in buffer. For rose bengal and eosin Y, edge-oriented pyrolytic graphite electrodes were first polished sequentially using wet 600 grit SiC paper, 1-μm alumina (Buehler, Lake Bluff, NY), and 0.3-μm alumina (Buehler), with intervening and final sonications in Milli-Q water. Pyrolytic graphite electrodes were pretreated by cycling the potential between +1 V and −1 V for 5 min at a scan rate of 500 mV/sec. The working electrode area was 0.32 cm^2; the reference electrode was Ag/AgCl (1.00 M KCl); the auxiliary electrode was a Pt wire. Experiments were conducted with argon-sparged ambient (23 ± 2°C) solutions in the dark. Concentrations were 500 μM for rose bengal and eosin Y and ≈40 μM for cercosporin. Formal potentials ($E^{\circ\prime}$) were taken to be $(E_{pc} + E_{pa})/2$, where E_{pc} and E_{pa} are the CV cathodic and anodic peak potentials (21). All potentials are reported vs. the normal hydrogen electrode, which is −0.23 V vs. Ag/AgCl.

Chemicals. Cercosporin was extracted from cultures of *C. kikuchii* (3). Rose bengal was from Aldrich. Eosin Y, *N*-phenylglycine, sodium dithionite, GSH, sodium ascorbate, and L-cysteine were from Sigma.

RESULTS

Microscopic Analysis of Cercosporin Reduction. Preliminary experiments (using a 450- to 490-nm excitation filter coupled with a 520-nm barrier filter) with cercosporin-producing cultures of *C. kikuchii* demonstrated that cercosporin crystals, which accumulate in the culture medium during growth, fluoresced the expected red color, whereas the hyphae fluoresced yellow-green. Hyphae of non-cercosporin-producing cultures did not fluoresce at these wavelengths. When cercosporin-producing cultures of *C. kikuchii* were viewed with a bandpass filter specific for reduced cercosporin (530 filter), hyphae fluoresced green, indicative of reduced cercosporin (Fig. 2*A*). Only yellow fluorescence (again indicative of reduced cercosporin) was seen with the 605 bandpass filter (Fig. 2*B*). Identical results were obtained when cercosporin was added to non-cercosporin-producing cultures of *C. kikuchii* and *C. nicotianae* (data not shown). When cercosporin-producing *C. kikuchii* cultures were killed by exposure to chloroform vapor, heat, or UV light, the hyphae fluoresced red when viewed with the 605 filter (Fig. 2*C*) and showed no

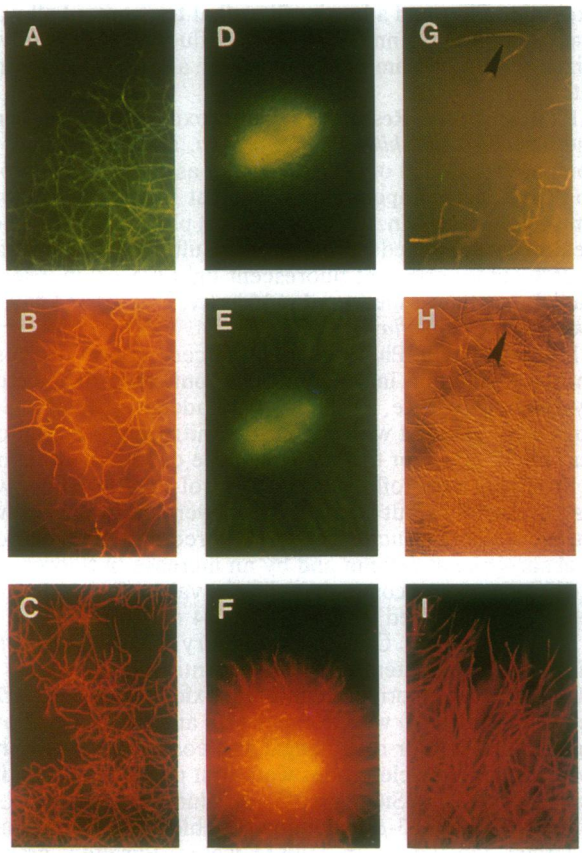

FIG. 2. Fluorescence microscopy of *C. kikuchii*, *C. nicotianae*, and *Asp. flavus* incubated with photosensitizers. (*A*) Cercosporin-producing culture of *C. kikuchii* viewed with the 530 filter (specific for reduced cercosporin); green fluorescence of hyphae indicates cercosporin reduction. (*B*) Same culture viewed with the 605 filter; yellow fluorescence indicates cercosporin reduction. (*C*) Same culture killed by exposure to chloroform vapor and viewed with the 605 filter; red fluorescence indicates lack of cercosporin reduction. (*D*) *Asp. flavus* incubated with cercosporin and viewed with the 530 filter; reduced cercosporin was present only at colony center. (*E*) Same as *D* except photographed with both brightfield and fluorescence, allowing visualization of nonfluorescing hyphae. (*F*) *Asp. flavus* viewed with the 605 filter; reduced cercosporin (yellow fluorescence) was present in the colony center but not (red fluorescence) at the actively growing periphery. (*G* and *H*) Fluorescence and interference contrast views of a non-cercosporin-producing culture of *C. nicotianae* incubated with eosin Y; lack of fluorescence indicates reduction of eosin Y. Arrowheads point to same hyphal strand in *G* and *H*. (*I*) *C. nicotianae* incubated with rose bengal; red fluorescence from rose bengal indicates lack of reduction of this compound. (A–C and G–I, ×50; D–F, ×25.)

fluorescence with the 530 filter (data not shown), indicating that only living hyphae were capable of maintaining cercosporin in a reduced state.

Other fungi were also tested for cercosporin-reducing ability. *Alt. alternata* (cercosporin-resistant) was capable of reducing cercosporin as indicated by fluorescence visualized with the 530 filter (Fig. 3*A*). By contrast, *N. crassa* and *Asp. flavus* (cercosporin-sensitive) had only limited ability to reduce cercosporin. When viewed with the 530 filter, green fluorescence (reduced cercosporin) was detected only in the centers of colonies of *Asp. flavus*; hyphae at the actively growing colony periphery did not fluoresce (Fig. 2 *D* and *E*). These observations were confirmed with the 605 filter, where yellow fluorescence (reduced cercosporin) was visible only in the colony center, and hyphae at the growing edge fluoresced red (Fig. 2*F*). When viewed with the 530 filter, hyphal tips of *N. crassa* occasionally fluoresced (Fig. 3 *B* and *C*); most of the mycelium, however, showed no fluorescence.

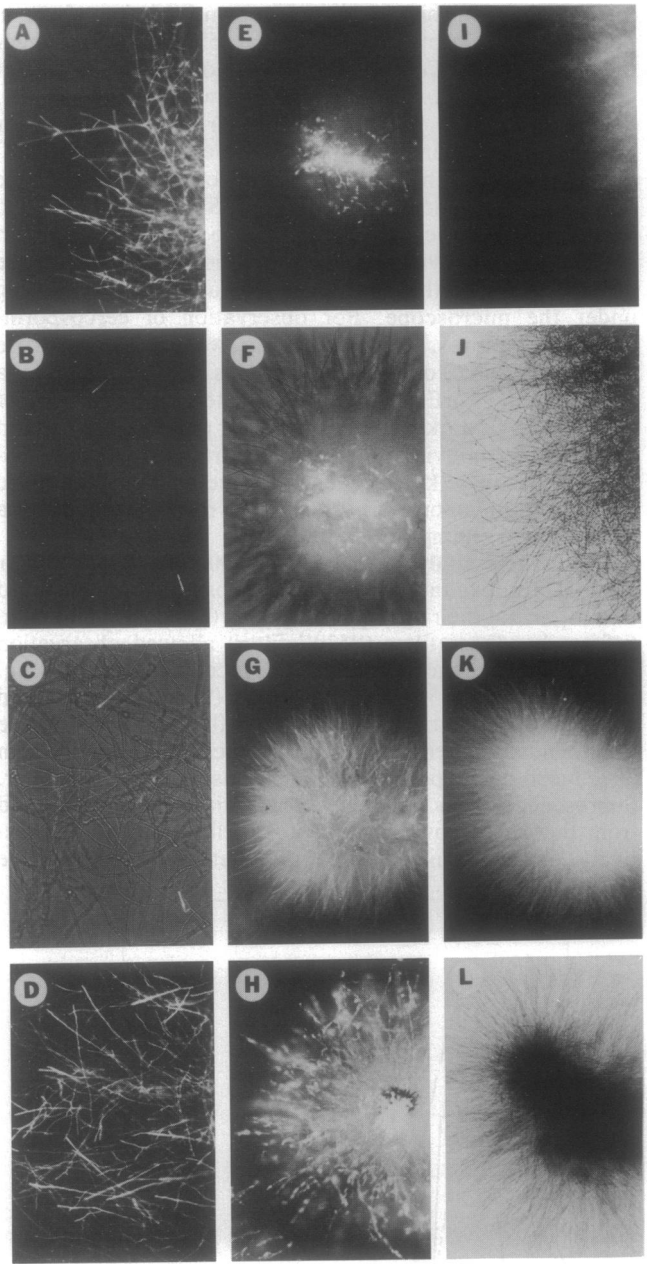

FIG. 3. Fluorescence microscopy of cercosporin-resistant and sensitive fungi. (*A*) *Alt. alternata* (cercosporin-resistant) incubated with cercosporin and viewed with the 530 filter; uniform fluorescence indicates cercosporin reduction. (*B*) *N. crassa* (cercosporin-sensitive) incubated with cercosporin and viewed with the 530 filter; limited fluorescence indicates minimal reduction of cercosporin. (*C*) Same as *B* except photographed with both brightfield and fluorescence, allowing visualization of nonfluorescing hyphae. (*D*) *N. crassa* incubated with cercosporin and ascorbate and viewed with the 530 filter; uniform fluorescence indicates cercosporin reduction. (*E* and *F*) Fluorescence and fluorescence–brightfield images of *Asp. flavus* (cercosporin-sensitive) incubated with cercosporin, mounted in a cysteine solution, and viewed with the 530 filter; distribution of fluorescence (reduced cercosporin) does not differ from controls. (*G*) *Asp. flavus* incubated with cercosporin and cysteine and viewed with the 530 filter; fluorescence (reduced cercosporin) is seen throughout the colony. (*H*) *Asp. flavus* incubated with cercosporin and ascorbate and viewed with the 530 filter; fluorescence (reduced cercosporin) is seen throughout the colony. (*I* and *J*) Fluorescence and brightfield images of a non-cercosporin-producing culture of *C. nicotianae* incubated with eosin Y; lack of fluorescence indicates reduction of eosin Y. (*K* and *L*) Fluorescence and brightfield images of *Asp. flavus* incubated with eosin Y; hyphal fluorescence indicates lack of eosin Y reduction. (A and D–L, ×25; B and C, ×50.)

Effects of Reducing Agents on Cercosporin Reduction. Reducing agents such as cysteine, GSH, and ascorbate protect cercosporin-sensitive fungi against cercosporin phototoxicity, although they are unable to reduce cercosporin directly (22). *N. crassa* and *Asp. flavus* were either grown for 16–18 hr in the presence of reducing agents or were mixed with the agents just prior to viewing. *N. crassa* reduced cercosporin when incubated with ascorbate (Fig. 3*D*, compare with control in Fig. 3*B*) or GSH (data not shown). *Asp. flavus* did not reduce cercosporin when cysteine was added before viewing (Fig. 3 *E* and *F*) but did reduce it when cultures were incubated with cysteine overnight (Fig. 3*G*). The opposite was true with ascorbate: *Asp. flavus* reduced cercosporin when mixed with ascorbate prior to viewing (Fig. 3*H*) but not when incubated with it for 16–18 hr (data not shown). Thus both fungi appeared capable of reducing cercosporin in the presence of reducing agents, although the specific agent and incubation conditions were critical.

Photoreduction. Detection of cercosporin reduction required that cultures be mounted under a coverslip and irradiated with light. Some photosensitizers undergo photoreduction under lowered oxygen tensions. To ensure that reduction was not an artifact of the experimental conditions, cercosporin was tested for its ability to be photoreduced in the presence of the medium and reducing agents used in the microscope assays but in the absence of oxygen and the fungus. Cercosporin photoreduction was monitored both by a loss of absorbance at 572 nm and by the appearance of fluorescence at 522 nm (see Fig. 1 for spectra). Photoreduction of rose bengal (10 μM) in the presence of *N*-phenylglycine was used as a control for the apparatus; rose bengal was efficiently photoreduced (86% decrease in A_{549}) under the assay conditions. No photoreduction was detected by changes in either absorbance or fluorescence when cercosporin was incubated in water or in PDB in the absence of reducing agents (data not shown). Upon the addition of cysteine, ascorbate, and GSH, limited but consistent cercosporin photoreduction was detected, but only by monitoring changes in fluorescence. As a percent of the total fluorescence emitted by dithionite-reduced cercosporin, cercosporin photoreduction was 2.4%, 1.5% and 0%, respectively, for cysteine, GSH, and ascorbate in water, and 0.8%, 1.7%, and 1.4%, respectively, for cysteine, GSH, and ascorbate in PDB.

Phototoxicity of Other $^1O_2$-Generating Photosensitizers. Both rose bengal and eosin Y strongly inhibited the growth of the cercosporin-sensitive fungi *N. crassa* and *Asp. flavus* (Table 1). Inhibition by rose bengal was comparable to that by cercosporin, but eosin Y was less phototoxic. As with cercosporin, *Asp. flavus* was more sensitive to the photosensitizers than was *N. crassa*. *C. kikuchii* was resistant to eosin Y, was slightly inhibited by cercosporin, and was strongly inhibited by rose bengal.

Fungal Reduction of Eosin Y and Rose Bengal. Reduction of eosin Y and rose bengal by dithionite converted them to colorless, leuco forms with no detectable fluorescence (data not shown). Thus reduction of these compounds by fungi was assayed microscopically by a lack of fluorescence. When *C. nicotianae* was incubated with eosin Y, only occasional fluorescence of hyphae was seen, and actively growing hyphae did not fluoresce (Fig. 2 *G* and *H* and Fig. 3 *I* and *J*). In contrast, *Asp. flavus* displayed uniform colony fluorescence in the presence of eosin Y (Fig. 3 *K* and *L*), demonstrating that lack of fluorescence of *Cercospora* hyphae was not due to lack of staining by eosin Y but presumably due to reduction of eosin Y to a leuco, nonfluorescent form. *C. nicotianae* was not able to reduce rose bengal; hyphae displayed uniform red fluorescence in its presence (Fig. 2*I*).

Table 1. Percent inhibition of fungal growth by singlet oxygen-generating photosensitizers

Fungus	% inhibition		
	Cercosporin	Rose bengal	Eosin Y
C. kikuchii	20 ± 5	59 ± 14	7 ± 5
N. crassa	72 ± 2	72 ± 2	53 ± 6
Asp. flavus	92 ± 9	96 ± 5	84 ± 8

Radial growth under constant light (75 $\mu E \cdot m^{-2} \cdot sec^{-1}$) was compared with growth on medium lacking photosensitizers. All compounds were tested at 10 μM. Values are mean ± SD of four experiments.

Electrochemical Reduction of Photosensitizers. For cercosporin, well-behaved diffusional CV responses were obtained at indium–tin oxide electrodes in neutral 20% acetone solutions. The $E^{\circ\prime}$ was determined to be -0.14 ± 0.01 V (vs. the normal hydrogen electrode) in a saturated ($\approx$40 μM) pH 7.5 solution containing 4.4 mM potassium phosphate, 75 mM KCl, and 20% acetone. Details of the voltammetry will appear elsewhere (R.A.C. and E.F.B., unpublished data). The reductive electrochemistry of rose bengal and eosin Y was more complicated than that of cercosporin, as it involved irreversible chemical reactions following electron transfer. The exact nature of these reactions is unclear but probably involves halide loss (23) and/or ring opening (24). For eosin Y, the following chemical reactions resulted in loss of the reverse-scan anodic peak (E_{pa}), thereby precluding the assignment of $E^{\circ\prime}$ by CV. For rose bengal, the $E^{\circ\prime}$ was determined to be -0.72 ± 0.03 V vs. the normal hydrogen electrode, which is $\approx$0.6 V negative of cercosporin for the same solution conditions. Eosin Y was easier to reduce than rose bengal, as its reduction peak was consistently located 50–100 mV positive of the corresponding rose bengal peak. This observation is in agreement with previous results obtained with a dropping mercury electrode (25). Thus, the ease of reduction at neutral pH is in the order cercosporin >> eosin Y > rose bengal.

DISCUSSION

The results support the hypothesis that cercosporin in contact with hyphae of cercosporin-resistant fungi is in a reduced form. Fluorescence typical of reduced cercosporin was observed from cultures of both *C. kikuchii* and *C. nicotianae* as well as from *Alt. alternata*. By contrast, actively growing hyphae of the cercosporin-sensitive fungi *N. crassa* and *Asp. flavus* did not reduce cercosporin, nor did nonviable cultures of *Cercospora*. We were unable to document efficient photoreduction of cercosporin in the absence of fungal hyphae, even under anaerobic conditions and in the presence of reducing agents. Our results thus indicate that reduction of cercosporin is an active function specific to cercosporin-resistant fungi. Furthermore, cercosporin reduction by resistant fungi appears to be localized to the fungal hyphae, since crystals of photoactive cercosporin accumulate in the medium around hyphae during fungal growth in culture.

In this study, green fluorescence visualized through a 515- to 545-nm bandpass filter was taken to indicate the presence of reduced cercosporin. Our justifications for this conclusion are as follows. (*i*) Reduced cercosporin fluoresces at these wavelengths, whereas cercosporin and its known derivatives do not. (*ii*) *Cercospora* species exhibit greater cell surface reducing capability (assayed by the ability of fungi to reduce tetrazolium dyes of differing redox potential) than do sensitive fungi (22). (*iii*) As shown in this study, incubation of sensitive fungi with reducing agents resulted in the presence of the green-fluorescing form of cercosporin. Thus, although we cannot rule out the possibility of an alternative chemical modification, all information available is consistent with the hypothesis that fluorescence visualized with the 530 filter is due to reduced cercosporin.

Previously we demonstrated that cercosporin can be reduced by strong reductants such as dithionite or zinc dust, but the reduced molecule readily reoxidizes upon aeration or extraction away from the reducing agent (26). Stable, reduced, methylated and acetylated derivatives were synthesized and shown to absorb less light, produce significantly less $^1O_2$, and be less phototoxic than were oxidized derivatives. Taken together, our studies indicate that cercosporin-resistant fungi can reduce and detoxify cercosporin in close contact with fungal hyphae. Further, this reduction is reversible, allowing for the accumulation of photoactive cercosporin needed for infection of host plants.

To our knowledge, reversible, reductive detoxification has not previously been reported as a cellular defense mechanism against photosensitizers. Reducing agents are known to act against photosensitizers and active oxygen species, but their mode of action has been attributed to mechanisms independent of photosensitizer reduction. For example, thiol compounds can react directly with $^1O_2$ (27), act as antioxidants (28), and protect against radiation and high oxygen concentrations (29). Also, membrane dehydrogenases are known that protect against oxidant injury by reducing lipid hydroperoxides (30). Previous work showed that reducing agents (cysteine, GSH, and ascorbate) that did not directly reduce cercosporin were able to protect sensitive fungi against cercosporin phototoxicity (22). Although these results could suggest that these agents protect by interfering with oxidant damage rather than by cercosporin reduction, results from the present study indicate that these agents can allow for cercosporin reduction by sensitive fungi, consistent with our model of protection by reduction and detoxification of the cercosporin molecule.

Further support for the reduction hypothesis comes from our studies on rose bengal toxicity to *Cercospora*. Quantum yields of $^1O_2$ for cercosporin and rose bengal are similar: 0.81 for cercosporin (measured in deuterated benzene; ref. 10) and 0.80 for rose bengal (measured in reverse micelles; ref. 31). Thus in the photoactive (nonreduced) state, cercosporin and rose bengal should have similar toxicity, a conclusion supported by the observation that these compounds are equally toxic to *Asp. flavus* and *N. crassa*. If the presence of a reducing environment acted exclusively by protecting against the toxic effects of $^1O_2$ rather than by preventing its formation by reducing cercosporin, we would not expect to see differential toxicity of these two compounds.

Cercosporin and rose bengal differ significantly, however, in their ease of reduction. Microscopic observation indicated that *Cercospora* species were not capable of reducing rose bengal, although they appeared to be able to reduce the structurally similar eosin Y, to which they are resistant. Electrochemical measurements support this interpretation. Cercosporin is by far the easiest of these compounds to reduce, and rose bengal is the most difficult. Thus, the ordering of these compounds by their *in vitro* reducibilities is consistent with the observed reduction by fungi and with our hypothesis of reductive detoxification.

We conclude that *Cercospora* species protect themselves against cercosporin by the reduction and detoxification of the cercosporin molecule in contact with fungal hyphae. Cercosporin that diffuses away from the fungal cell spontaneously reoxidizes to the photoactive form needed for infection of host plants. This mode of resistance is uniquely adapted to an organism that utilizes a photosensitizer to survive in nature. Spontaneous reoxidation of cercosporin in the absence of a strong reducing agent eliminates the need for a specific reactivation mechanism. Membrane oxidoreductase systems that transfer reductants across the plasma membrane are a normal component of cells (30). It seems likely that normal growth of *Cercospora* species results in the maintenance of a reducing environment sufficient for cercosporin reduction. This would explain how these organisms synthesize and grow in the presence of cercosporin without any measurable decrease in growth. Our results also suggest that resistance by photosensitizer reduction is not limited to cercosporin alone, since *Cercospora* species appear capable of reducing eosin Y, to which they are resistant, but cannot reduce the structurally similar rose bengal, which is the only $^1O_2$-generating photosensitizer identified thus far that is toxic to these fungi. Studies on other organisms that synthesize and utilize photosensitizer molecules are needed to determine whether photosensitizer reduction has general applicability or is limited to these cercosporin-producing fungi.

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