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Inactivation of *Neurospora crassa* Conidia by Singlet Molecular Oxygen Generated by a Photosensitized Reaction

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Photodynamic damage of *Neurospora crassa* conidia was studied in the presence of the photosensitizing dye, toluidine blue O. Conidia which germinated to form colonies decreased in number as irradiation time became longer. The photoinactivation of conidia was suppressed by azide, bovine serum albumin, and histidine, and was stimulated in deuterium oxide. Wild-type conidia were less sensitive to the irradiation than albino conidia. In the wild type, carotenoid-enriched conidia were more resistant against the lethal damage than the conidia which contained small amounts of carotenoids. These results suggest that singlet molecular oxygen causes photodynamic lethal damage to *N. crassa* conidia and that singlet molecular oxygen is quenched by endogenous carotenoids.

Intense visible light causes lethal damage to many organisms. The photodynamic damage is striking in the presence of both photosensitizing dyes and molecular oxygen. Singlet molecular oxygen ($^1O_2$) is considered to be one of the factors which cause photodynamic damage. Methylene blue (6, 7, 25), toluidine blue (1, 2, 9), acridine orange (11, 24), and eosin Y (5) have been used to generate $^1O_2$ under irradiation. The photodynamic inactivation induced by $^1O_2$ has been studied extensively in *Sarcina lutea* (18, 19) and *Saccharomyces cerevisiae* (5, 9, 11).

Blanc et al. (4) reported the photodynamic inactivation of *Neurospora crassa* conidia. However, a clear explanation has not been made for the mechanism of the inactivation. The present study shows the effect of $^1O_2$ formed by photosensitized reaction on conidial germination of *N. crassa*. Results obtained suggest the participation of $^1O_2$ in the photodynamic damage and the function of carotenoids as a quencher of $^1O_2$ in vivo.

MATERIALS AND METHODS

The strains of *N. crassa* used were a carotenoid-containing wild-type 4A (FGSC 821) and an albino strain isolated from a cross between STA4 (FGSC 262) and LT2a (a multiply marked stock having *al-2*) which grew well on a synthetic minimal medium (3).

Cultures to obtain conidia were grown on the minimal medium. Inoculated cultures were incubated for 2 days at 26°C in the dark. Then the cultures were allowed to stand for 8 h under room light at room temperature; 7 to 10 days after inoculation, conidia were harvested with 50 mM potassium phosphate buffer (pH 7.0) and filtered through a layer of glass wool to remove hyphae. The conidial suspension was centrifuged at low speed, and the sediment was washed once. Conidia obtained were suspended in the same buffer and counted with a Thoma blood counter. The reaction mixture (3 ml) contained 5×10^5 to 7×10^5 conidia per ml and 10 μM toluidine blue O in the 50 mM potassium phosphate buffer. The pH or pD [−log (deuterium ion)] of this buffer was adjusted to 7.0, and pD was determined with a glass electrode by applying a correction of +0.4 pH unit (16).

Conidial suspensions were irradiated at 28°C in a test tube having a diameter of 13 mm. For control experiments, samples were wrapped in aluminum foil. Irradiation light was provided by a 1,000-W projector, and the light was passed through a yellow glass filter (>520 nm; Corning CS 3-68). Light intensity on the surface of the samples was 55,000 lx.

After irradiation, the reaction mixture was diluted appropriately and plated onto sorbose medium (28) in petri dishes to form colonies. After 2 or 3 days, colonies were counted.

In some experiments, the carotenoid content of the conidia was controlled. Harding et al. reported that the formation of carotenoid pigments in *Neurospora* was dependent on light (8). Accordingly, culture flasks were wrapped in aluminum foil and incubated throughout in the dark to lessen the carotenoid content of the conidia, which was determined as follows. Conidia harvested with 50 mM potassium phosphate buffer (pH 7.0) were washed once with a large volume of cold distilled water and lyophilized. Carotenoids of lyophilized conidia were extracted with methanol and acetone successively. Judging from the color of the extracted conidia, almost all of the carotenoids seemed to have been extracted from the conidia. The methanol and acetone extracts were combined and dried on a rotary evaporator in a vacuum. The dried carotenoids were dissolved in hexane to measure absorption spectra. Three absorbance peaks at 444, 470, and 495 nm were obtained independent of the carotenoid content of the conidia. Carotenoid content was estimated by measuring the middle peak (14). The amount of pro-

tein was determined by the method of Lowry et al. (15).

Toluidine blue O and deuterium oxide (D_2O, 99.75%) were purchased from Merck & Co., Inc.; bovine serum albumin and histidine were from Wako Chemical Co. (Osaka); and azide was from Katayama Chemical Co. (Osaka).

RESULTS AND DISCUSSION

Inactivation of *N. crassa* conidia by $^1O_2$. Table 1 shows the effect of toluidine blue O on the survival (colony formation) of wild-type conidia irradiated or kept in the dark (control) for 60 min. In the lower concentrations of toluidine blue O (less than 10 μM), the dye did not affect the survival of conidia either in the light or dark. A 10 μM toluidine blue O concentration showed no effect on the survival of non-irradiated conidia, but it decreased the survival of irradiated ones remarkably. At 30 μM, even the survival of non-irradiated conidia decreased. In the following experiments, 10 μM toluidine blue O was used to examine whether $^1O_2$ induced the inactivation of conidia.

Figure 1 shows the effect of the active oxygen scavenger, azide (21), on the light-induced inactivation of wild-type conidia in the presence of toluidine blue O. In the absence of azide, half of the conidia were inactivated by 20 min of irradiation. As the concentration of azide increased, a longer time of irradiation was required to inactivate half of the conidia. At 5 mM azide, no remarkable inactivation of conidia was observed even with 80 min of irradiation. Other active oxygen scavengers, histidine (1 mM) (17) and bovine serum albumin (2.5 mg/ml) (17), also protected the conidia from damage. In the reagents examined, azide has been generally used as a $^1O_2$ quencher. At the concentrations of azide used here (3 and 5 mM), azide seems to be mainly functioning as a $^1O_2$ quencher but not as a radical scavenger (cf. 12). Therefore, we consider that $^1O_2$ is involved in the photodynamic damage. If $^1O_2$ participates in the photodynamic inactivation of conidia in the presence of toluidine blue O, D_2O must stimulate the inactivation because of the longer lifetime of $^1O_2$ in D_2O than in water. The effect of D_2O on the photodynamic inactivation of wild-type conidia was examined (Fig. 2). D_2O stimulated the rate of photodynamic inactivation by about twofold. The lifetime of $^1O_2$ in 90% D_2O (the concentration of D_2O in the present experiment) is about five times longer than that in water (20, 23).

TABLE 1. *Effect of toluidine blue O on the survival of wild-type N. crassa conidia*[a]

Concn of toluidine blue O (μM)	Survival (%)[b]	
	Non-irradiated[c]	Irradiated[d]
0	100	100
0.3	100	94
1	—	106
3	98	102
10	110	16
30	65	0

[a] Basic reaction mixture (3 ml) contained 5×10^5 to 7×10^5 conidia per ml in 50 mM potassium phosphate buffer (pH 7.0).

[b] Numbers indicate normalized values, expressed as a percentage of the number of colonies formed in the absence of toluidine blue O. —, Not examined.

[c] Samples were kept in the dark for 60 min.

[d] Samples were irradiated for 60 min.

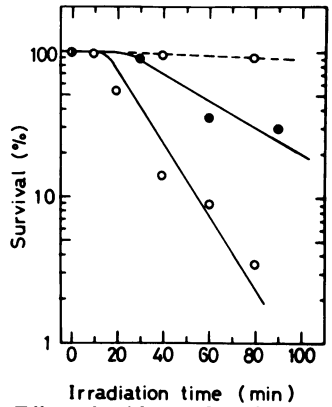

FIG. 1. *Effect of azide on photodynamic inactivation of wild-type conidia. Basic reaction mixture (3 ml) contained 5×10^5 to 7×10^5 conidia per ml and 10 μM toluidine blue O in 50 mM potassium phosphate buffer (pH 7.0). Symbols:* (○——○) *no addition;* (●——●) *3 mM azide;* (○- - -○) *5 mM azide. In the absence of irradiation, 90-min-incubated conidia with 3 mM azide and 80-min-incubated conidia with 5 mM azide showed about 90 and 70% conidial survival, respectively, compared with the dark control of 0 mM azide. Each curve was obtained by calculations based on the respective dark control.*

The results in Fig. 1 and 2 led to the conclusion that $^1O_2$ formed by the photosensitized reaction caused the inactivation of *N. crassa* conidia. As to the mechanism of cell inactivation by $^1O_2$, it is probably lipid peroxidation in the plasma membrane, resulting in irreversible changes in membrane properties. The $^1O_2$-induced lipid peroxidation of membrane lipids was reported in chloroplasts (27), mitochondria (29), and microsomes (22). The erythrocyte lysis accompanying lipid peroxidation was also observed (10). Under the present experimental conditions, $^1O_2$ generated outside of the conidia seemed to bring about lethal damage on conidia because bovine serum albumin, which may be impermeable to plasma membrane, protected conidia from $^1O_2$-induced damage.

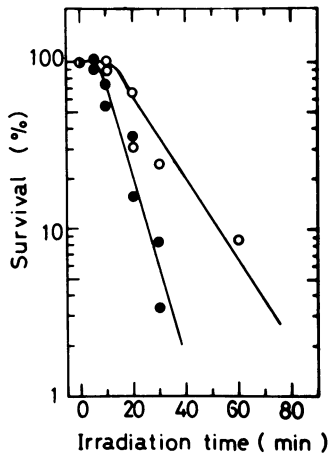

FIG. 2. *Time courses of photodynamic inactivation of wild-type conidia in water and 90% D_2O. Basic reaction mixture (3 ml) contained 5 × 10^5 to 7 × 10^5 conidia per ml and 10 μM toluidine blue O in 50 mM potassium phosphate buffer prepared with water or 90% D_2O (pH or pD 7.0). Symbols: (○——○) in water; (●——●) in 90% D_2O. In the absence of irradiation, 90% D_2O had little effect on the survival of N. crassa conidia.*

Although we consider here $^1O_2$ to be the main factor causing the photodynamic lethal damage of *Neurospora* conidia, the possibility that other species of active oxygen (O_2^-, OH·) are formed during the photodynamic reaction to induce lethal damage of *Neurospora* conidia is not excluded.

Possible function of endogenous carotenoids as $^1O_2$ quenchers. Because carotenoids have been reported to be efficient quenchers of $^1O_2$ in vitro (1, 2, 6), it is possible to consider that function for carotenoids in vivo (13). To make sure of the function of endogenous carotenoids in *N. crassa* conidia, we compared the rates of $^1O_2$-induced inactivation in wild-type and albino conidia (Fig. 3). Albino conidia were inactivated more rapidly than wild-type conidia. The mean irradiation time to reduce by half the number of surviving conidia was 23.8 min in wild-type (seven trials) and 11.7 min in albino conidia (four trials). Azide also protected albino conidia from photodynamic inactivation.

The photodynamic inactivation of carotenoid-enriched conidia (wild type) was examined and was compared with that of carotenoid-impoverished conidia of the same wild-type strain. The results (Table 2) showed that carotenoid-enriched conidia were more resistant to irradiation than were carotenoid-impoverished ones. Although the differences did not appear terribly great here, the numbers were significantly different ($0.05 > P$). The carotenoid contents in carotenoid-enriched and carotenoid-impoverished condidia were 3.0 and 9.3 μg/g of protein, respectively (mean values of four trials). The effect shown in Table 2 was also observed in another series of experiments in which carotenoid-enriched conidia showed 100% survival of the dark control after 15 min of irradiation, whereas carotenoid-impoverished conidia showed only 44%.

Results in Fig. 3 and Table 2 suggest that carotenoids contained in *N. crassa* conidia play a role in the quenching of $^1O_2$, since the decrease in the number of surviving conidia was caused by $^1O_2$ (Fig. 1 and 2). Protection by endogenous

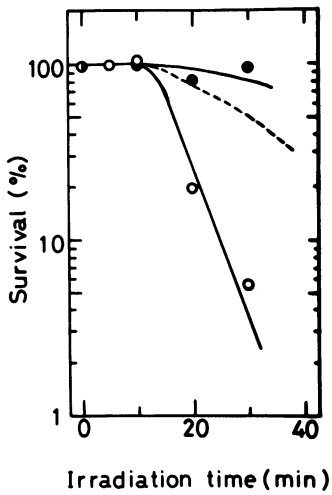

FIG. 3. *Time courses of photodynamic inactivation of albino conidia. Basic reaction mixture was the same as described in the legend to Fig. 1. Symbols: (○——○) no addition; (●——●) 3 mM azide; (-----) wild-type conidia. The wild-type curve is from Fig. 1. The methanol-acetone extract of lyophilized albino conidia did not reveal any significant peaks in the wavelength from 400 to 600 nm.*

TABLE 2. *Photodynamic inactivation of the carotenoid-enriched and -impoverished conidia*[a]

Irradiation time (min)	Colonies/plate[b]		*t* test
	Carotenoid-enriched conidia	Carotenoid-impoverished conidia	
0	23.6 ± 4.10 (4)	25.0 ± 1.82 (4)	
10	25.3 ± 3.09 (4)	19.3 ± 3.68 (3)	$0.1 > P > 0.05$
20	18.7 ± 4.04 (4)	12.3 ± 2.16 (4)	$0.05 > P$
30	11.0 ± 0.81 (4)	7.5 ± 1.11 (3)	$0.05 > P$

[a] Basic reaction mixture was the same as in Fig. 1. There was no difference in conidial survival in the dark control between carotenoid-enriched and -impoverished conidia.

[b] Values are expressed as mean ± standard deviation. Values in parentheses indicate number of plates.

carotenoids has been reported in studies on the photodynamic lethal damage of *S. lutea* (18, 19). That damage may be induced by $^1O_2$. In chloroplasts, β-carotene quenched $^1O_2$ generated by photosynthetic electron transfer (26). However, as suggested by Krinsky (13), we must also consider the possibility that carotenoid is capable of scavenging O_2^- formed by a photodynamic reaction in *N. crassa* conidia. Direct evidence for the function of endogenous carotenoids of *N. crassa* conidia as a $^1O_2$ quencher is open to further studies.

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