Induction of "Petite" Mutants of Yeast, *Saccharomyces cerevisiae*, by Photodynamic Action of Acriflavine

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Treatment of yeast cells with acriflavine followed by illumination with fluorescent lamps resulted in extensive "petite" induction in addition to rapid cell inactivation and nuclear gene mutation. There was no petite induction or cell inactivation in the un-illuminated cells. Such photobiological damage induced by acriflavine was not observed under deoxygenated conditions, such as in the presence of NaN_3, which is a scavenger of singlet oxygen ($^1O_2$). Photodynamic treatment of yeast cells did not cause marked changes in the CsCl sedimentation profile of mitochondrial DNA. These results showed that the petite induction and the cell inactivation after acriflavine treatment are mainly due to type II photodynamic action.

Keywords——petite induction; photodynamic action; acriflavine; yeast; *Saccharomyces cerevisiae*; sectored colony; cell inactivation; singlet oxygen; sodium azide; cesium chloride equilibrium density gradient centrifugation

"Petite" mutants of yeast, *Saccharomyces cerevisiae*, are induced by a variety of chemicals.[1] Most of them (typified by acriflavine) are active only in growing cells whereas the others (typified by ethidium bromide) are effective in both growing and non-growing cells. Induction of petite mutation in the growing cells is attributed to the selective inhibition[2] of mitochondrial DNA replication, which results in the substantial deletion of mitochondrial DNA in the progeny cells. Petite induction in the non-growing cells has been accounted for in terms of the degradation of mitochondrial DNA by a specific DNase[3] stimulated by the mutagens.

In this communication we describe the induction of petites by photodynamic action of acriflavine. Illumination (visible light) of acriflavine-sensitized cells brought about marked petite induction in addition to rapid cell inactivation. It is suggested that these photobiological activities of acriflavine are mainly due to type II photodynamic action.

Experimental

Strain and Cultures——A haploid yeast strain of *Saccharomyces cerevisiae* DP1 1B/517 (α, his_1, trp_1, ρ^+, ω^+, C^R) was used. Cells were grown for 18 h (late exponential phase) in YPD medium [1% yeast extract (Difco), 2% peptone (Difco) and 1% dextrose] at 30 °C with shaking. Cells were harvested, washed three times in 1/15 M phosphate buffer (pH 7.0), sonicated to separate clumping cells and adjusted to a cell density of 10^6/ml.

Photodynamic Treatment of Yeast Cells——Resuspended cells (10^6/ml) were incubated with 1 μM acriflavine (Aldrich) at 30 °C for 60 min in the dark. Aliquots of sensitized cell suspension were taken into Thunberg tubes and illuminated with fluorescent lamps (National, 15 W × 4, 7 cm distance), while the remaining cell suspension was kept in the dark as a control. The deoxygenated state was obtained by repeated evacuation and filling with pure nitrogen gas of the Thunberg tubes. Cell suspensions, illuminated or un-illuminated, were spread on plates of YPD medium after suitable dilution. After 3 d of incubation at 30 °C in the dark, the frequencies (%) of petite and sectored colonies were determined by the tetrazolium-overlay method[4] as described in the previous report.[5] Colonies which were completely white were scored as petite, and colonies which had distinct portions of white and red were scored as sectored. Survivors were calculated from the number of colonies relative to the dark control.

Induction of Nuclear Gene Mutation——Treated cells were plated on Yeast Nitrogen Base (Difco, w/o amino acid, dehydrated) medium supplemented with methionine and histidine but not tryptophan. The Trp^+ revertants that

grew on this medium were counted after a 7-day incubation at 30 °C in the dark.

³H-Labeling of Mitochondrial DNA——Cells in the late exponential phase were suspended (final 10^6 cells/ml) in 25 ml of YPD medium and cultured for 16 h at 30 °C. Cycloheximide (final 200 μg/ml) was added to the vessel to inhibit the labeling of nuclear DNA.[6] After a 20-min incubation, ³H-uracil (New England Nuclear, [6-³H]uracil, 22 Ci/ mmol) was added to a final concentration of 20 μCi/ml. Five ml of labeled cell suspension was removed and centrifuged. Cells were washed three times with SET (1.3 M sorbitol, 0.1 mM EDTA and 10 mM Tris-HCl, pH 7.4). The remaining suspension was centrifuged, washed three times with the buffer and resuspended in 200 ml of phosphate buffer at a cell density of 10^7/ml.

Photodynamic Treatment of Labeled Cells——Labeled cells were treated with 1 μM acriflavine at 30 °C for 60 min in the dark. Ten ml aliquots of suspensions were transferred to 10 Thunberg tubes. Five of them were exposed to fluorescent lamps for 30 min and the other five for 60 min as described earlier. Fifty ml of the remaining suspension was taken as the dark control. Samples were removed from each suspension, diluted adequately and plated onto plates of YPD medium to check the frequencies (%) of petites and survivors.

Spheroplast Formation and Preparative CsCl Density Gradient Centrifugation——Cell pellets were washed well with buffer and SET, then resuspended in 20 μl of Zymolyase solution [Zymolyase 60000 (Kirin Brewery Co.), 2 mg; 2-mercaptoethanol, 30 μl; 0.1 M EDTA, 40 μl; SET, 1 ml] and incubated at 30 °C for 60 min. The cells were washed well with cold SET and lysed for 30 min at 37 °C with 20 μl of 10% Sarcosyl (Nikkol). Lysates were diluted with 1.7 ml of SSC (0.15 M NaCl, 0.015 M Na-citrate·3Na, pH 7.0) and treated with bovine pancreatic RNase (Miles, Grade II) at 37 °C for 30 min. Cell lysates (1.5 ml each) were mixed with 0.5 ml of *Micrococcus lysodeikticus* DNA (Miles) and 6.0 ml of CsCl solution [CsCl (Wako), 22.7 g; SSC, 40.3 ml; $\rho = 1.778$ g/ml] and centrifuged in a type 65 fixed-angle rotor using a Hitachi 65 P ultracentrifuge (35 krpm for 65 h at 20 °C). After centrifugation, samples were collected in fractions of drops each (about 130 μl) from the bottom.

Determination of Radioactivity and Buoyant Density——The fractions were incubated overnight at 30 °C after the addition of 0.1 ml of 5% NaOH in order to hydrolyze RNA. Then bovine serum albumin (1 mg/ml, 0.1 ml) and trichloroacetic acid (20%, 0.8 ml) were added to each fraction and the mixture was stored overnight at 4 °C. Precipitates were collected on glass fiber filters (GF/C Whatman) by filtration. The filters were subsequently rinsed with cold 5% TCA and ethanol. Radioactivities of the dried filter papers were measured with an Aloka LSI-661 liquid scintillation counter using a toluene scintillator [2,5-diphenyloxazole, 4.0 g, 1,4-bis[2-(5-phenyloxazolyl)]benzene, 0.1 g; toluene, 1000 ml]. To determine the density, aliquots (100 μl) were removed from every fifth tube and weighed. The buoyant density of each fraction was corrected based on the position of the density marker ($\rho = 1.7318$ g/ml of *M. lysodeikticus* DNA). In order to identify the fractions containing *M. lysodeikticus* DNA, distilled water (3 ml each) was added to fractions 11 through 19, and the absorbances at 260 nm were measured.

Results

Petite Induction and Cell Inactivation by the Illumination of Acriflavine-Sensitized Cells

Yeast cells treated with 1 μM acriflavine (AF) for 60 min at 30 °C were illuminanted with fluorescent lamps (Fig. 1). The frequency of sectored colonies reached a maximum (62.4%) after illumination for 10 min, indicating that cells which would form sectored colonies rapidly decreased during prolonged illumination. In contrast, the frequency of petite induction began to increase after a 10-min illumination and reached a plateau value of 85% after a 20-min illumination. Cell inactivation occurred upon illumination for 10 min or more, and survivors decreased exponentially (25.4% at 20 min and 5.6% at 30 min). No petite induction or cell inactivation was observed in un-illuminated cells. These results indicated that AF induces petites in addition to cell inactivation by photodynamic action.

Photodynamic Action of Various Concentrations of Acriflavine

The photodynamic action of AF was examined at various concentrations. As shown in Fig. 2, the rate of petite induction was the highest (92.4%) at 1 μM AF and then decreased with increasing concentration, while the percentage of sectored colonies increased with increase of AF concentration. Survivors decreased with the increasing concentration of AF.

Efficiency of Photodynamic Action at Different Cell Densities

Cells were suspended in 1/15 M phosphate buffer (pH 7.0) at cell densities of 10^6, 10^7 and 10^8/ml, and treated with AF. Aliquots were taken into Thunberg tubes and illuminated with fluorescent lamps. As shown in Table I, notable petite and sectored colony inductions
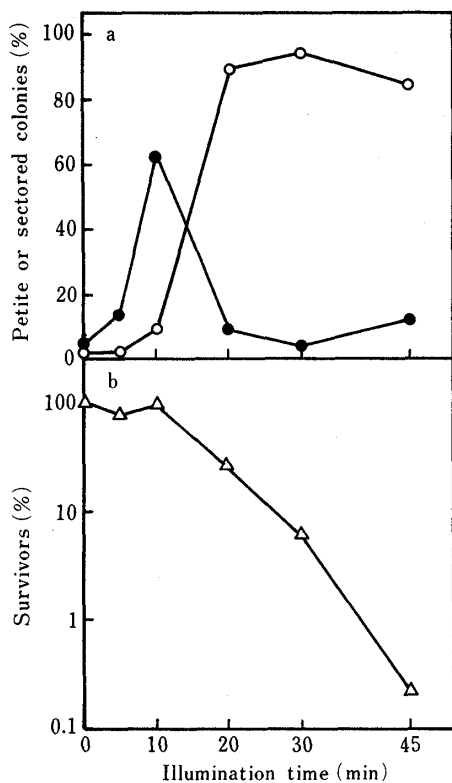
Fig. 1. Time Course of Petite Induction and Cell Inactivation by the Illumination of Acriflavine-Sensitized Cells

Yeast cells were incubated with 1 μM AF at 30 °C for 60 min in the dark. Aliquots were placed in Thunberg tubes and illuminated by fluorescent lamps (National, 15 W × 4, 7 cm distance), while the remaining cells were kept in the dark as a control.

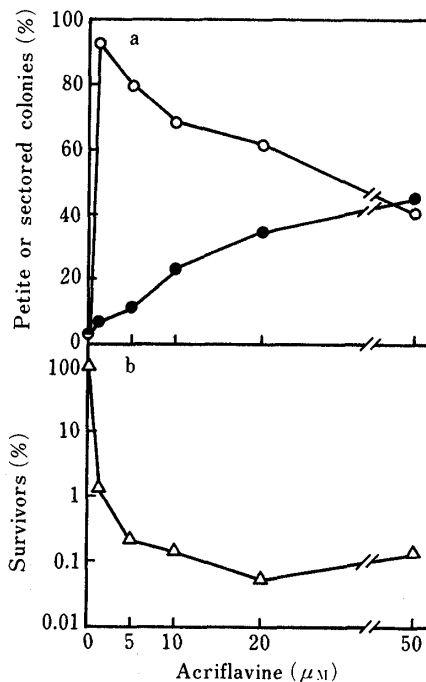Petite colonies, ○—○; sectored colonies, ●—●; survivors, △—△.

Fig. 2. Photodynamic Action of Various Concentrations of Acriflavine

Cells were incubated with various concentrations of AF at 30 °C for 60 min. Aliquots were placed in Thunberg tubes and illuminated for 30 min. Symbols were the same as in Fig. 1.

TABLE I. Efficiency of Photodynamic Action at Various Cell Concentrations

Cell concn. (cells/ml)	AF (μM)	Petite (%)	Sector (%)	Survivors (%)
10^6	1	$92.4 \pm 2.8^{a)}$	6.0 ± 2.7	1.6
	10	67.8 ± 3.1	23.2 ± 1.2	0.16
	50	40.2 ± 1.5	44.7 ± 5.7	0.15
10^7	1	61.3 ± 3.4	38.5 ± 1.8	19.4
	10	52.1 ± 2.8	43.4 ± 1.8	0.78
	30	22.0 ± 7.8	33.6 ± 4.8	0.60
10^8	10	3.5 ± 2.0	2.9 ± 1.9	77.9
	50	2.4 ± 0.9	7.3 ± 2.6	62.5
	100	3.5 ± 0.6	3.8 ± 1.7	98.5

a) Values are means ± S.D. of four plates.

together with cell inactivation were observed at cell densities of 10^6 and 10^7 cells/ml. All cell damage was greater at 10^6 than at 10^7 cells/ml. At a given cell density, increasing concentration of AF caused a decrease of petite induction and an increase of cell inactivation

TABLE II. Photodynamic Action of Acriflavine in the Presence
of NaN_3 or in the Deoxygenated State

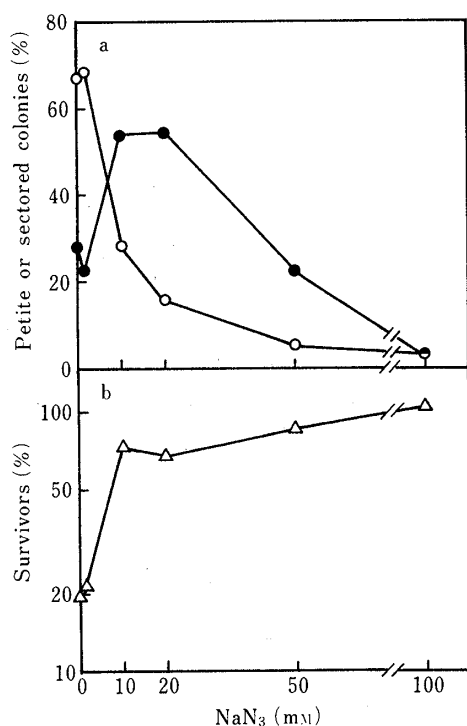| Conditions of illumination aerobic/anaerobic/NaN_3	min	Petite (%)	Sector (%)	Survivors (%)
Aerobic	0	$3.5 \pm 1.2^{a)}$	8.6 ± 2.8	100
	30	67.0 ± 0.7	28.1 ± 1.7	19.4
	60	86.0 ± 1.3	13.8 ± 1.4	3.1
Aerobic + NaN_3 50 mM	30	5.6 ± 2.1	21.9 ± 6.6	84.7
Anaerobic (deoxygenated)	30	1.4 ± 0.8	7.8 ± 3.0	133.9
	60	2.1 ± 1.3	5.0 ± 1.1	127.4

a) Values are means $\pm$ S.D. of four plates.

Fig. 3. Effects of NaN_3 on the Photodynamic
Action of Acriflavine

Cells sensitized with $1 \mu M$ AF were placed in
Thunberg tubes containing various amounts of NaN_3
and illuminated for 30 min. Symbols were the same as
in Fig. 1.

as in Fig. 2. The percentage of sectored colonies increased with the decrease of petite induction in a limited range. These results suggested that sectored colonies represent an intermediate stage in petite induction. Neither petite induction nor cell inactivation was observed at 10^8 cells/ml.

Photodynamic Action of Acriflavine in the Presence of NaN_3 or in the Deoxygenated State

Sensitized cells were illuminated in the presence of 50 mM NaN_3 or in the deoxygenated state (Table II). Illumination in the deoxygenated state did not bring about any petite induction or cell inactivation, while illumination under aerobic conditions caused marked petite induction and cell inactivation. The addition of NaN_3, which is known to scavenge singlet oxygen ($^1O_2$),[7] prevented these photobiological effects of AF. The effects of NaN_3 were examined further, as shown in Fig. 3. NaN_3 did not affect the photodynamic action at 1 mM. The rate of petite induction in the presence of 10 mM NaN_3 was 28.2% as compared to 67.0% in the control without NaN_3, and at the same time the survival rate recovered to 71.8% as compared to 19.4%. The petite induction and cell inactivation decreased with increasing concentration of NaN_3, and at 100 mM the petite induction and cell inactivation were

TABLE III. Induction of Nuclear Gene Mutation

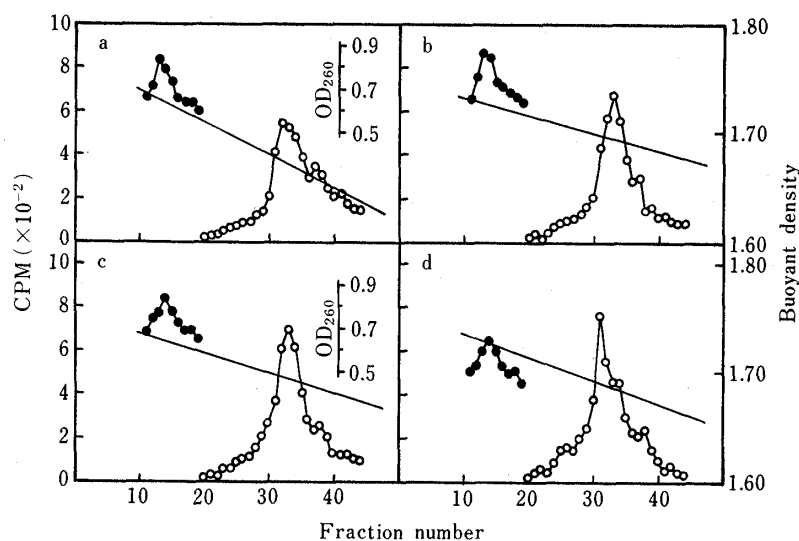| Acriflavine (μM)	Trp$^+$ Revertants (%)		Ratio[a]	Survivors (%)
	Illuminated	Un-illuminated		
0	2.66×10^{-5}	2.73×10^{-5}	0.97	97.4
10	1.31×10^{-3}	2.59×10^{-5}	50.6	5.8

a) Illuminated/un-illuminated.

Fig. 4. CsCl Equilibrium Density Gradient Centrifugation

Yeast cells were incubated with ^{3}H-uracil in the presence of cycloheximide as described in Experimental. An aliquot of labeled cells was removed as a control (a) and the remaining cells were sensitized with 1 μM AF at 30 °C for 60 min. One-third of the sensitized cells was used as the dark control (b). One-third was illuminated for 30 min (c) and the remaining one-third for 60 min (d). Each cell was subjected to Zymolyase treatment and CsCl equilibrium density gradient centrifugation. Radioactivity, O—O; OD_{260}, ●—●; buoyant density, ——.

completely prevented.

Induction of Nuclear Gene Mutation by the Photodynamic Action of Acriflavine

The rate of reversion from tryptophan auxotroph to prototroph was examined. As shown in Table III, the rate of reversion to Trp$^+$ in AF-sensitized cells was increased about fifty times by illumination, while that of control cells was unchanged. These results indicated that photodynamic mutagenesis of AF is effective not only on the mitochondrial DNA but also on the nuclear DNA.

Change of Isopycnic CsCl Sedimentation Profiles of Mitochondrial DNA by Photodynamic Action of Acriflavine

The effect of AF upon the CsCl sedimentation profile of mitochondrial DNA was examined. As shown in Fig. 4, the sedimentation profile of AF-sensitized cells (b) was essentially identical to that of unsensitized cells (a). Peak radioactivity of mitochondrial DNA was observed around fraction 30 to 40 and there was no peak of nuclear DNA, suggesting that cycloheximide inhibited the incorporation of ^{3}H-uracil into nuclear DNA completely. The illumination of sensitized cells for 30 min (c) or 60 min (d), where the frequencies of petites were 55.4% (c) and 92.0% (d), did not bring about any marked change in the bouyant density

of mitochondrial DNA.

Discussion

Acridine compounds act as photosensitizers in a variety of organisms.[8] Acriflavine,[9] proflavine,[9,10] acridine orange[7,9-11] and acridine yellow[10] inactivate yeast cells and also induce gene conversion by photodynamic action. 8-Methoxypsoralen[12] and thiopyronine,[13] which are potent photosensitizers, are known to induce petites. Some acridines induce petites under growth conditions in the absence of visible light.[14] We showed that acriflavine induces petites even under non-growth conditions by photodynamic action.

Photodynamic cell damage is suggested to be induced *via* a type I (covalent binding of dyes to the targets) or type II (singlet oxygen-mediated reaction process).[15] The induction of gene conversion or cell inactivation by acridine dyes and light is mediated by the singlet oxygen.[7,8a,9] In the present work, the petite induction by the photodynamic action of AF was also proved to be singlet oxygen-mediated, because petites were not induced in the deoxygenated state or in the presence of NaN_3, which scavenges singlet oxygen (Table II, Fig. 3).

The percent incidence of petites decreased with increasing concentration of AF, and the number of suvivors at 10 or 20 μM was much less than at 1 μM (Fig. 2). Similar findings of low petite induction at high AF concentration are shown in Table I. From these observations, we think that the process of petite induction by the photodynamic aciton may be as follows; AF molecules are intercalated between base pairs of mitochondrial DNA, and illumination causes their excitation, followed by the generation of singlet oxygen under aerobic conditions. At high concentration, the dye molecules bound to membranes (cytoplasmic and mitochondrial) or present in the cytoplasm or mitochondrial matrix may absorb light, so that less energy is used to induce petites. This speculation is in accordance with the suggestion that the cell inactivation due to photodynamic action is mainly due to damage to membranes[9,16] on the cell surface. The failure of petite induction and cell inactivation at 10^8 cells/ml (Table I) is explained by shadowing blocking of light from some cells by others.

8-Methoxypsoralen[17] or trimethoxypsoralen[18] is known to bind DNA covalently and show type I photodynamic action. In *S. cerevisiae*, interstrand crosslinks were induced in both nuclear and mitochondrial DNA after treatment with 8-methoxypsoralen and near-ultraviolet (UV) irradiation.[17c] If extensive double-strand scission (which results in small fragments of different GC content) occurred, the sharp peak of the mitochondrial DNA would be broadened. As shown in Fig. 4, the photodynamic treatment of yeast cells did not cause any marked change in the sedimentation profile of mitochondrial DNA.

To clarify the molecular events occurring in petite induction by the photodynamic action of AF, the possibility of single-strand scission should be examined by alkaline sucrose gradient centrifugation.

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