Singlet Oxygen Production and Photobiological Effects of Pinacyanol Chloride on Yeast *Saccharomyces cerevisiae*

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Photobiological activities of pinacyanol chloride (PC), which is known as a non-intercalating dye, were investigated. Irradiation of PC-sensitized yeast cells in the dark brought about marked decrease of survival and induction of "petites" which are respiration-deficient mutants caused by partial loss of mitochondrial deoxyribonucleic acid. Nuclear mutation represented by reversion from Trp− to Trp+ was also induced by photodynamic action of PC. This fact suggested phototoxic dyes are not necessarily intercalated for inducing mitochondrial and nuclear mutation. Singlet oxygen production was determined in the photoirradiated PC solution by electron spin resonance spectrometry. Photobiological effects of PC might be brought about mainly by a type II photodynamic mechanism.

Keywords — pinacyanol chloride; photodynamic action; type II; yeast; *Saccharomyces cerevisiae*; petite induction; cell inactivation; nuclear mutation; singlet oxygen; ESR detection

**Introduction**

Pinacyanol chloride (PC) is a quinoline dye (Fig. 1) which has been used as a diagnostic dye in obstetrics and optical sensitizer in photography. It effectively induces "petite mutants"1) which are respiration-deficient mutants of yeast caused by partial loss of mitochondrial deoxyribonucleic acid (mitochondrial DNA). This dye is active only in growing cells but not in non-growing cells like acriflavine (AF).2) PC has been reported to interact on the surface of double helical DNA parallel to axis, perpendicular to the plane of base pair,3) while AF is intercalated between base pairs of DNA.4) In the previous reports we clarified AF, a potent petite inducer only in growing yeast cells without light, induced petites effectively under non-growing condition by photodynamic action.5) The process of petite induction is supposed to be as follows: Intercalated AF molecules into mitochondrial DNA are energized by photoirradiation and excite oxygen molecules to singlet oxygens. These active oxygen species attack DNA molecules from inside and produce changes which result in the petite mutation. Therefore it is of interest to determine whether or not PC, a non-intercalating dye, brings about mutation(s). From this respect the photobiological activities of PC on yeast *Saccharomyces cerevisiae* were investigated.

**Materials and Methods**

**Strains and Cultures** — A haploid strain of *Saccharomyces cerevisiae* DP11B/517 (α, his1, trp1, ρ+, ω+, Cr) was used. Cells were grown for 18 h (late logarithmic phase) in YPD medium [1% yeast extract (Difco), 2% peptone (Difco) and 1% dextrose] at 30 °C with shaking. Cells were washed three times with 1/15 M phosphate buffer (pH 7.0), sonicated to separate clumping cells, and adjusted to a cell density of

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Fig. 1. Chemical Structure of Pinacyanol

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Photobiological Activity of Pinacyanol

10⁸/ml in the same buffer.

Petite Induction by Phototreatment of PC — Resuspended cells (10⁸/ml) were incubated with 1 µM PC at 30 °C for 60 min in the dark. Aliquots of PC-sensitized cells were taken into Thunberg tubes and irradiated with fluorescent lamps (National, 15 W × 4, 8000 lx), while the remaining cell suspensions were kept in the dark as a control. Cell suspensions irradiated or not, were spread on YPD plates after suitable dilution. After 3 d of incubation at 30 °C in the dark, the frequencies (%) of petite colonies were determined by the tetrazolium-overlay method⁶ as described in previous reports.⁷ Survivals (%) were calculated from the number of colonies relative to the dark control.

Induction of Nuclear Gene Mutation — Sonicated yeast cells were suspended in the same phosphate buffer described earlier at a cell density of 5 × 10⁷/ml and incubated with 10 µM PC at 30 °C for 60 min in the dark. Cell suspensions were diluted with the same buffer to a concentration of 10⁷/ml, transferred into Thunberg tubes and irradiated as described earlier. Cells were collected by centrifugation and resuspended ed in 1.0 ml of buffer (10⁸ cells/ml). An aliquot of the cell suspension (0.1 ml containing 10⁷ cells) was 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-d incubation at 30 °C in the dark. Treated cells were also plated on YPD medium to determine the percent survival.

Absorption Spectroscopy — Absorption spectra were measured using a Hitachi U-3210 spectrophotometer as described in previous report.⁸

Electron Spin Resonance (ESR) Detection of Singlet Oxygen — Singlet oxygen production in irradiated PC solution was detected with ESR according to the previous reports.⁹

Chemicals — PC was purchased from Eastman Kodak. Euflavine was purified from commercial acriflavine (Aldrich) that contains proflavine (3,6-diaminoacridine) by the procedure of Gupta et al.¹⁰ and used as a reference. All other chemicals were of reagent grade.

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Fig. 2. Cell Inactivation and Petite Induction by Photodynamic Action of PC

Yeast cells (10⁸/ml) were incubated with 1 µM PC at 30 °C for 60 min in the dark. An aliquot of cell suspension was removed and irradiated for 30 min with fluorescent lamps. △, survival; ○, petite (irradiated); ●, petite (dark control).

Fig. 3. Photobiological Effects of Different Concentrations of PC

Yeast cells were incubated with different concentrations of PC (0.25—2.00 µM) in the dark. Cells were irradiated for 30 min by fluorescent lamps: △, survival; ○, petite.
TABLE I. Induction of Nuclear Mutation (Trp⁻→Trp⁺ Reversion) by Photodynamic Action of PC

<table>
<thead>
<tr>
<th></th>
<th>Irradiated</th>
<th>Not-irradiated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony number/YNB (−) plate</td>
<td>128 ±47</td>
<td>16 ±4</td>
</tr>
<tr>
<td>Colony number/YNB (+) plate</td>
<td>226 ±26</td>
<td>43 ±7</td>
</tr>
<tr>
<td>(Dilution rate)</td>
<td>(10⁻⁵)</td>
<td>(10⁻⁵)</td>
</tr>
<tr>
<td>Reversion rate</td>
<td>5.66 × 10⁻⁴</td>
<td>3.72 × 10⁻⁷</td>
</tr>
<tr>
<td>Irradiated/not-irradiated</td>
<td>15.2</td>
<td></td>
</tr>
</tbody>
</table>

a) Yeast nitrogen base (YNB) medium depleted of tryptophan. b) YNB medium supplemented with tryptophan (10 mg/1).

Results

Petite Induction and Cell Inactivation by Photodynamic Action of PC

Yeast cells treated with 1 μM PC for 60 min at 30 °C in the dark were irradiated with fluorescent lamps (Fig. 2). Cell inactivation was observed at 10 min irradiation and survivals decreased exponentially (63.7% at 20 min, 16.4% at 30 min and 4.8% at 40 min). Marked petite induction was also observed. Frequency of petite induction began to increase after a 10-min irradiation and reached a plateau value after 20-min irradiation. These results showed that PC is an active photosensitizer both in cell inactivation and petite induction.

Photodynamic Action of Various Concentrations of PC

![Hypochromic Spectral Changes of PC Solutions](image)

PC was dissolved in 1/15 m phosphate buffer, pH 7.0 to make a 10 μM solution. Each solution was irradiated by fluorescent lamps (National, 15 W × 4, 8000 lx) in Thunberg tube.

![ESR Detection of Singlet Oxygen Production in Irradiated PC Solution](image)

PC (10⁻⁵ M) and TEMP (2 × 10⁻² M) were dissolved in 1/15 m phosphate buffer (pH 8.0) and irradiated with a UV lamp (Toshiba SHL-100 UV-2 type) for 18 min from 3 cm distance. ESR spectra were recorded as described previously.90
Cells treated with 0.25—2.0 \( \mu \text{M} \) PC were irradiated for 30 min to examine minimum effective concentration. As shown in Fig. 3, survival decreased to 50% remarkably at 0.5 \( \mu \text{M} \) and 0.5% at 1.0 \( \mu \text{M} \) of PC. Petite induction was also efficient (50.1%) at 0.5 \( \mu \text{M} \) and almost reached plateau (\( > 80\% \)) at 1.0 \( \mu \text{M} \).

**Induction of Nuclear Gene Mutation**

As shown in Table I, irradiation of the PC-sensitized cells brought about a marked increase (15.2 fold) of the reversion rate from tryptophan auxotroph to prototroph. These results suggested that PC interacts with nuclear DNA and damages by photodynamic action.

**Spectral Changes of PC Solutions Following Irradiation**

PC is a dichromatic dye and has two absorption maxima in the visible light area as shown in Fig. 4. When the PC solution (10 \( \mu \text{M} \) in 1/15 M phosphate buffer, pH 7.0) was irradiated by fluorescent lamps in Thunberg tube, a sharp hypochromic shift was observed.

**ESR Detection of Singlet Oxygen**

Singlet oxygen production in PC solution by irradiation was determined by ESR. As shown in Fig. 5, singlet oxygen produced in the irradiated PC solution is captured by 2,2,6,6-tetramethyl-4-piperidine (TEMP) to form 2,2,6,6-tetramethyl-4-piperidone-N-oxyl (TEMPO). TEMPO is a stable nitroxide radical detectable by ESR spectrometry. In order to detect singlet oxygen production, an aqueous solution of TEMP was irradiated by a UV lamp in the presence of PC. Characteristic nitroxide signals were detected in irradiated PC solutions, whereas there was no signal in irradiated TEMP solution devoid of PC. Time courses of TEMPO production by PC-photosensitization were examined at \( 10^{-4} \) and \( 10^{-5} \) M PC. As shown in Fig. 6, relative intensity of TEMPO signal at \( 10^{-5} \) M PC was maximum at 2 min of irradiation and declined with further irradiation and disappeared at 5 min. At \( 10^{-4} \) M relative intensity of signals were higher than \( 10^{-5} \) M through irradiation time but disappeared after 5 min.

**Discussion**

AF and PC are potent inducers of petite\(^1\)\(^,\)\(^2\) respiration-deficient mutant of yeast caused by mitochondrial mutation. They are effective only in growing cells but not in non-growing cells. AF was reported to be intercalated reversely between the base pairs on double helical DNA\(^3\) while PC is interacted with DNA in a non-intercalating manner,\(^4\) namely PC adheres on the surface of double helix parallel to the axis and perpendicular to the plane of base pairs. In the previous works acridine dyes including AF were shown to be a potent photodynamic petite inducer\(^5\) in non-growing yeast cells. In this communication photodynamic mutagenic activities of PC, a non-intercalating dye, were investigated. Both petite induction and reverse mutation from tryptophan auxotroph to prototroph were induced effectively by the photodynamic action of PC in addition to the severe cell inactivation (Fig. 2, 3, Table I). ED\(_{50}\) value of petite inducing activity of PC (0.5 \( \mu \text{M} \)) obtained from Fig. 3 was almost equivalent to that of AF. These results suggested that “intercalation” is not indispensable for photosensitizers to induce mutations. Irradiation of PC solution brought about a sharp hypochromic shift (Fig. 4). This fact suggested PC may act through type II mechanism mediated by singlet oxygen as shown previously\(^6\)\(^,\)\(^7\) in AF.
photosensitization. In fact the production of singlet oxygen was detected in PC solution by ESR spectrometry (Fig. 5). These results suggested PC is a potent photosensitizer which inactivates yeast cells and induces both nuclear and mitochondrial mutations like AF.

References


6) M. Ogur, R. S. John and S. Nagai: Tetrazolium overlay technique for population studies of respiration deficiency in yeast, Science, 125, 928—929 (1957).


