Near-UV-induced absorbance change and photochemical decomposition of ergosterol in the plasma membrane of the yeast *Saccharomyces cerevisiae*

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When cells of the yeast *Saccharomyces cerevisiae* were exposed to near-UV (300–400 nm), their absorption spectra changed slightly within the range 220–300 nm with increasing dosage. Difference spectra, calculated by subtracting the curve recorded in cells exposed to near-UV from the curve of unexposed cells, decreased with increasing dosage over a broad band with peaks at 272, 282 and 295 nm and a shoulder at 265 nm. These peaks were in agreement with the absorption maxima of ergosterol, which is one of the major components of the plasma membrane of yeast. Near-UV radiation induced a simultaneous decrease in absorption spectra and reduction of ergosterol content in the plasma membrane. Photochemical decomposition of ergosterol by near-UV radiation was revealed *in vivo*, although ergosterol is generally known to be photoconverted to previtamin D₂ industrially by UV radiation *in vitro*. In order to remove photosensitizers, liposomes were prepared from phospholipids and glycolipids, with or without ergosterol from purified yeast plasma membranes. Liposomal ergosterol in the orientated state was photochemically decomposed by near-UV radiation but ergosterol in the disorientated state in a homogeneous solution was not. Near-UV radiation also induced a decrease in activity of membrane-bound ATPase. Dose-response curves for the reduction of ATPase activity were similar to that for decomposition of ergosterol, suggesting that near-UV caused membrane function damage.

Keywords: near-UV, ergosterol, membrane damage, plasma membrane, *Saccharomyces cerevisiae*

INTRODUCTION

It is well known that near-UV radiation induces inactivation of the cell and repression of cell growth and multiplication (Jagger, 1981). DNA damage accompanied by formation of pyrimidine dimers (Boyce & Howard-Flanders, 1964), DNA single-strand breaks (Rosenstein & Ducore, 1983) and generation of activated oxygen species by photosensitizers (Piette *et al.*, 1986) is directly induced by UV radiation and causes serious damage in all organisms. However, near-UV radiation results in little direct DNA damage, such as formation of pyrimidine dimers (Tyrrell, 1973).

Near-UV radiation clearly reduces some functions of the plasma membrane, such as active transport (Robb *et al.*, 1978; Ascenzi & Jagger, 1979; Sharma & Jagger, 1981),

ion transport (Siegel & Corn, 1974) and its ability to act as a permeability barrier (Ito & Ito, 1983). In our previous study, the dose-response curve for survival of yeast cells exposed to near-UV was similar to that for disruption of the permeability barrier and for uptake of amino acids, indicating that reduction of these membrane functions was related to inactivation of the cell (Arami *et al.*, 1993). These phenomena suggest that the target for membrane damage by near-UV is a component(s) within the plasma membrane and that photochemical alteration of the target may result in damage of membrane function, which plays an important role in normal intracellular activity.

The plasma membrane is composed mainly of proteins and lipids. The function of the plasma membrane is maintained and regulated by the delicate balance of

constituents in the membrane. Changes in the constituents disrupt this balance, inducing reduction of various membrane functions and finally leading to inactivation of the cell. In this study, we examine a constituent(s) in the plasma membrane altered by near-UV. When a cell suspension and membrane fraction of the yeast *Saccharomyces cerevisiae* were exposed to near-UV at various dosages, absorbance changes were detected as difference spectra resulting in a decrease over a broad band with peaks at 272, 282 and 295 nm and a shoulder at 265 nm. It was indicated that the decrease in absorbance resulted from photochemical decomposition of ergosterol in the plasma membrane.

METHODS

Culture and irradiation procedure. *Saccharomyces cerevisiae* IS66-4C α was grown in the dark at 26 °C for 20 h in YPD medium containing 1% yeast extract, 2% Bactopeptone and 2% glucose (w/v) with continuous shaking at 125 r.p.m. until in stationary phase. Yeast cells were harvested by centrifugation and washed twice with distilled water. The cells were suspended in distilled water to a final concentration of 0.13 mg dry cell weight ml⁻¹ and pipetted into quartz cuvettes. Near-UV irradiation was carried out as described previously by Tada & Shiroishi (1982) with slight modifications. Cell suspensions were exposed to near-UV emitted from a fluorescent lamp (National FL20S BL-B; emission wavelength 300–400 nm) with continuous stirring and cooled with ice. Where nitrogen gas and air were bubbled through during irradiation, the cells were suspended in degassed distilled water which was prepared by sonication under reduced pressure. The flow rate of gases was 70 ml min⁻¹. Absorbance changes in cell suspensions exposed to near-UV at various dosages were measured with an automatic spectrophotometer (Shimadzu UV-3000). In order to detect slight changes of absorption spectra, difference spectra were calculated by subtracting the curve recorded in irradiated cells from the curve of intact control cells.

Ascorbate and α -tocopherol were used as radical scavengers. α -Tocopherol was dissolved in tetrahydrofuran/DMSO (1:1, v/v) and added to the yeast suspension at a final concentration of 1%. After incubation at 30 °C for 2 h in the dark, yeast cells were harvested by centrifugation. During irradiation by near-UV, yeast cells were suspended in distilled water containing 10 mM ascorbate at the final density described above.

Preparation of the plasma membrane. Yeast cells were suspended in 0.1 M Tris/HCl buffer (pH 7.2) containing 10 mM EDTA, 1.2 M sorbitol and 1 mg zymolyase ml⁻¹. The reaction mixture was incubated at 30 °C for 2 h and protoplasts were formed. After washing twice with the same buffer lacking zymolyase, protoplasts were burst by suspension in 0.1 M Tris/HCl buffer (pH 7.4). Impurities were removed by centrifugation at 2300 g for 5 min at 4 °C. The microsomal fraction was precipitated by centrifugation at 12000 g for 30 min at 4 °C. After the precipitate was washed twice with the latter buffer, the plasma membrane was collected using a density gradient with sucrose at 100000 g for 60 min at 4 °C. The layer containing the plasma membrane was determined by assay of Mg²⁺-ATPase, which was used as a marker enzyme. For determination of ATPase activity, the plasma membrane was suspended in a reaction mixture containing 50 mM Tris/HCl (pH 7.8), 3 mM MgCl₂ and 2 mM ATP to a final concentration of 1 mg protein ml⁻¹. After incubation at 30 °C for 10 min, the phosphate released from ATP was

determined as described by Fiske & Subbarow (1925). This layer was not contaminated by mitochondria since the ATPase activity was not inhibited in the presence of sodium azide.

Determination of ergosterol content. Ergosterol content was determined by the method of Arnezeder *et al.* (1989) with slight modifications. Samples exposed to near-UV were refluxed for 1 h in 5 ml 90% (v/v) ethanol containing 7-dehydrocholesterol used as an internal standard. After addition of 5 ml 50% (v/v) ethanol, the sample was filtered through a cellulose acetate filter (pore size 0.45 μ m). The filtrate was treated with a SEP-PAK C-18 cartridge and ergosterol was dissolved with 2-propanol. The solution containing ergosterol was analysed with HPLC apparatus attached to a Nucleosil ODS column (4.5 $\times$ 150 mm; 5 μ m particles). Using methanol/ethanol/water (86:10:4, by vol.) as the mobile phase, the solution was pumped through the column at a flow rate of 1.5 ml min⁻¹. Ergosterol and 7-dehydrocholesterol were monitored by the A₂₈₀.

Preparation of liposomes. The total lipids from plasma membranes of yeast cells were extracted as described by Allen & Good (1971). Plasma membranes were suspended in chloroform/methanol (1:2, v/v) and the suspension was vigorously stirred. After centrifugation at 1000 g for 5 min, the residue was re-extracted with chloroform/methanol (1:1, v/v). The chloroform layer was concentrated by a rotary vacuum evaporator. Total lipids were fractionated using silica gel C-100 column chromatography (13 $\times$ 2.0 cm) developing in the order chloroform, acetone and methanol, separating neutral lipids, glycolipids and phospholipids. Compositions of glycolipids and phospholipids were determined by two-dimensional TLC; the first development was with chloroform/methanol/water (65:25:4, by vol.) and the second development was with chloroform/methanol/ammonium hydroxide (63:35:5, by vol.).

Reconstructed liposomes were produced according to the method of Darszon *et al.* (1980). Fractions of glycolipids, phospholipids and commercial ergosterol were dissolved in a small amount of diethyl ether and transferred to an Erlenmeyer flask of 200 ml capacity. After evaporation of the solvent in a stream of nitrogen, distilled sterilized water was gently poured into this flask. Large liposomes were produced after sealing with nitrogen and maintained at room temperature for 24 h.

RESULTS

Near-UV-induced absorbance change

When yeast cell suspensions were exposed to near-UV at 10 J ml⁻¹, a slight change was observed in the absorption spectrum (Fig. 1a). In order to examine the detail of the spectral change, the difference spectra were calculated by subtracting spectra curves recorded in cells exposed to near-UV at various dosages from curves of intact cells. There was evidently a decrease over the broad band from 260 to 300 nm with increasing dosage and peaks were observed at 272, 282 and 295 nm (Fig. 1b). These three peaks were in excellent agreement with characteristic absorption maxima of ergosterol, which is one of the major components of the plasma membrane in fungi.

In the case of irradiation of the isolated plasma membrane, the difference spectra also had peaks at 272, 282 and 295 nm with a shoulder at 265 nm and were

Fig. 1. Effects of near-UV irradiation on the absorbance spectrum of intact cells of the yeast *S. cerevisiae*. (a) Absorption spectra before (solid line) and after (dotted line) irradiation at 10 J ml⁻¹ under ice cooling with continuous stirring. Cells were suspended in distilled water to a final concentration of 0.13 mg dry cell weight ml⁻¹. (b) Difference spectra were calculated by subtracting the curve recorded in yeast cells exposed to near-UV at a dosage of 1.25 (----), 2.5 (—), 5.0 (—) or 10 (—) J ml⁻¹ from the curve of intact cells. Curves were derived from single sets of results and were reproducible in repeated experiments.

extremely similar to the peaks observed in the cell suspension (Fig. 2). Moreover, an isosbestic point was found at 260 nm on both spectra. However, the difference spectra of the cell wall and cytoplasmic fractions were not similar to the cell suspension (data not shown). These facts indicated that spectral changes in the yeast cell suspension due to near-UV radiation originated from a change in the membrane fraction. The wavelength of the peaks in each spectra agreed with theoretical peaks of ergosterol in homogeneous solution, although the relative intensity differed. Since decreases in A_{282} occurred by altering the near-UV intensity and decreases were in direct proportion to the logarithms of the dosages in both cell suspension and the plasma membrane, the Bunsen-Roscoe reciprocity rule held under our experimental conditions.

Fig. 2. Effects of near-UV irradiation on the absorbance spectrum in a membrane suspension prepared from yeast cells. (a) Absorption spectra before (solid line) and after (dotted line) irradiation at 10 J ml⁻¹. Plasma membranes were suspended in distilled water to a final concentration of 0.1 mg protein ml⁻¹. (b) Difference spectra were calculated by subtracting the curve recorded in the membrane exposed to near-UV at a dosage of 1.25 (----), 2.5 (—), 5.0 (—) or 10 (—) J ml⁻¹ from the curves of the non-exposed membrane. Curves were derived from single sets of results and were reproducible in repeated experiments.

In order to examine whether near-UV-induced absorbance change depended on the oxygen concentration, suspensions of plasma membrane were exposed to near-UV with continuous bubbling of either nitrogen gas or air (Fig. 3). In samples with bubbled air, absorbance changes were similar to those in Fig. 2 but few changes were detected with bubbled nitrogen gas. These results suggested that absorbance changes were caused by photochemical decomposition of ergosterol dependent on oxygen coming from oxidative or peroxidative photolysis.

Effect of near-UV radiation on ergosterol content

After near-UV irradiation of cells and the plasma membrane, the ergosterol content was measured using HPLC. The content in both samples clearly decreased

Fig. 3. Dependence on oxygen of the absorbance change in the plasma membrane of yeast. During near-UV irradiation of the membrane at a dosage of 1.25 (----), 2.5 (—), 5.0 (— — —) or 10 (— · —) J ml⁻¹, air (a) or nitrogen gas (b) was bubbled into the suspension at a flow rate of 70 ml min⁻¹ under ice cooling. Curves were derived from single sets of results and were reproducible in repeated experiments.

Fig. 4. Decrease in content of ergosterol in intact yeast cells (a) and plasma membrane (b) by near-UV irradiation. Irradiation conditions were the same as in Figs 1 and 2. Each value represents the mean and standard error of results from three independent experiments.

with increasing dosage (Fig. 4). The relationship between near-UV-induced absorbance change and decrease in ergosterol content in intact cells and plasma

Fig. 5. Effects of radical scavengers on the decrease in ergosterol content in yeast cells by near-UV irradiation. α -Tocopherol dissolved in tetrahydrofuran/DMSO was added to yeast suspensions at a final concentration of 1% and incubated at 30 °C for 2 h in the dark. The yeast cells were exposed to near-UV with α -tocopherol and ascorbate (open bar). After incubation in the presence of the solvent vehicle, cells were exposed with (dotted bar) or without (single-hatched bar) ascorbate. Untreated cells were exposed as a control (double-hatched bar). Irradiation conditions were the same as in Fig. 1. Each value represents the mean and standard error of results from three independent experiments.

membranes was examined. A decrease in A_{282} was found to be directly proportional to the decomposition of ergosterol in the cell suspensions and plasma membrane, indicating that the absorbance change was reflected in a decreased ergosterol content. Yeast cells were then exposed to near-UV radiation in the presence of ascorbate and α -tocopherol, which were used as radical scavengers (Fig. 5). Regardless of the presence of these scavengers, ergosterol content decreased with increasing dosage. This finding indicated that ergosterol was

Fig. 6. Patterns of difference spectra of the orientated (a) and disorientated (b) state of ergosterol after near-UV irradiation. Liposomes were constructed with glycolipids and phospholipids extracted from plasma membranes of yeast in the presence (a) or absence (c) of ergosterol. An ethanol solution of ergosterol was used as the disorientated state (b). Each sample was exposed to near-UV at a dosage of 5.0 (dotted line) or 10.0 (solid line) J ml^{-1} under ice cooling. Curves were derived from single sets of results and were reproducible in repeated experiments.

directly photodecomposed by near-UV radiation without mediating radicals.

Decomposition of liposomal ergosterol by near-UV

It is well known that ergosterol and 7-dehydrocholesterol, which are used as starting compounds for production of vitamin D, are difficult to photo-convert into previtamin D above the 300 nm wavelength (Braun *et al.*, 1991). In spite of this fact, ergosterol was clearly decomposed by near-UV in this study. We measured absorbance changes of ergosterol due to near-UV radiation in the orientated and disorientated state. Photosensitizers generate activated oxygen species upon irradiation which cause widespread damage in various intracellular molecules. Therefore, we also prepared reconstructed liposomes to exclude the possibility that photodecomposition of ergosterol was mediated by photosensitizers.

When liposomes were constructed from phospholipids, glycolipids and ergosterol, then exposed to near-UV, the difference spectra drastically decreased with increasing dosage in a similar manner to the plasma membrane (Fig. 6a), although the spectra did not change in the liposome constructed without ergosterol (Fig. 6c). In the case of the disorientated state, a slight change similar to the pattern of the liposome without ergosterol was observed (Fig. 6b). In addition, the content of ergosterol apparently decreased in liposomes exposed to near-UV radiation but was constant in the ethanol solution (Fig. 7). During irradiation of liposomes with continuous bubbling of nitrogen gas, neither a decrease in absorption spectra nor a decrease in ergosterol content occurred (data not shown).

Fig. 7. Photochemical decomposition of liposomal (dotted bar) and homogeneous (closed bar) ergosterol after near-UV irradiation. Experimental conditions were the same as in Fig. 6. Each value represents the mean and standard error of results from three independent experiments.

Near-UV-induced reduction of membrane-bound ATPase activity

It is well known that the plasma membrane contains several varieties of ATPase which play important roles as ion pumps and antiports, and are essential for normal growth and maintenance of physiological activity in organisms. It was shown that near-UV radiation has an effect on ATPase activity and ergosterol content in the plasma membrane (Fig. 8). The ATPase activity was clearly reduced with increasing dosage, in parallel with

Fig. 8. Parallel decrease in content of ergosterol (a) and reduction of ATPase activity (b) after near-UV irradiation in plasma membranes. After irradiation, ergosterol was extracted from one aliquot and ATPase activity was assayed in another. Each value represents the mean and standard error of results from three independent experiments.

the decrease in content of ergosterol. Dose-response curves for the decrease in ergosterol content were very similar to that for reduction of ATPase activity, indicating that photodecomposition of ergosterol in the membrane might be closely related to the reduction of the activity of membrane-bound ATPase brought about by near-UV irradiation.

DISCUSSION

The detection of a primary photochemical reaction is significant since this reaction represents the starting point of a photobiological phenomenon. The measurement of near-UV-induced absorbance changes in yeast cells and plasma membranes resulted in a decrease over a broad band with peaks at 272, 282 and 295 nm and a shoulder at 265 nm. It was evident that this decrease in absorption spectra resulted, specifically, from photochemical decomposition of ergosterol through a photochemical reaction dependent on oxygen. In addition, intracellular photosensitizers might not be involved in photodecomposition of ergosterol since the degree of

absorbance change in reconstructed liposomes was comparable to that in plasma membranes.

It is well known that ergosterol is photoconverted to previtamin D₂, tachysterol and lumisterol by UV irradiation at 254 nm *in vitro* followed by heat conversion to vitamin D₂ (Havinga *et al.*, 1960; Havinga, 1973; Braun *et al.*, 1991). These compounds show absorption in the region 220–300 nm, since conjugated double bonds originating from the 5,7-sterol skeleton were maintained in this reaction (Braun *et al.*, 1991). We attempted identification of the photodegradation products of ergosterol by near-UV radiation *in vivo* but these intermediates were not detected by spectrophotometry, HPLC analysis or NMR (data not shown). However, ergosterol solubilized in SDS solution was photoconverted to previtamin D₂ following near-UV irradiation (unpublished data). The decrease in absorption spectra would imply cleavage of the 5,7-diene structure in ergosterol progressing with the photochemical reaction, since the broad band with specific peaks in the absorption spectrum of ergosterol is generally dependent on conjugated double bonds in the 5,7-sterol skeleton. This finding suggested that the mode of cleavage in membranes proceeded by a reaction distinct from the reaction of photoisomerization to previtamin D₂ *in vitro* as reported by Braun *et al.* (1991). However, there has been little investigation into the photodegradation product(s) of ergosterol after near-UV irradiation *in vivo*.

Ergosterol is one of the major membrane components and plays an important role in normal expression of membrane function in fungi. In practice, ergosterol affects the uptake of amino acids (Singh *et al.*, 1979), ion transport (Wagner *et al.*, 1980) and membrane-bound enzyme activity (Imbrie & Murphy, 1982). In addition, ergosterol is remarkably more useful than other sterols in stabilization of the membrane (Prasad, 1985). The activity of membrane-bound ATPase was clearly reduced in parallel to the decomposition of ergosterol. These facts further verify that ergosterol significantly affects physiological function and physicochemical properties of the plasma membrane. We reported previously that inactivation of the cell, so-called repression of cell growth, and membrane function, e.g. active transport and its ability to act as a permeability barrier, decreased sigmoidally with increasing dosage of near-UV radiation (Arami *et al.*, 1993). The dose-response curve for these phenomena was not in agreement with that for photodecomposition of ergosterol. However, a minimum level of ergosterol (approx. 25 % of normal content) is essential for yeast cell viability (Parks *et al.*, 1987; Leber *et al.*, 1995). Ergosterol content was maintained above this threshold level during the period of inactivation of the cell by near-UV under the conditions chosen from our experiments. Therefore, it is considered, based on these findings, that photodecomposition of ergosterol is a primary photochemical reaction and a trigger for inactivation of the cell and damage to membrane function following near-UV irradiation of fungi.

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