Reduction of ATPase activity accompanied by photodecomposition of ergosterol by near-UV irradiation in plasma membranes prepared from \textit{Saccharomyces cerevisiae}

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When plasma membranes prepared from the yeast \textit{Saccharomyces cerevisiae} were exposed to near-UV radiation, photodecomposition of ergosterol and reduction of ATPase activity occurred simultaneously. The $V_{\text{max}}$ for ATPase activity decreased markedly with increasing near-UV dosage while the $K_m$ value remained constant. When ATPase solubilized from the plasma membrane was exposed to near-UV, the activity remained constant irrespective of dosage, indicating that the ATPase molecule itself was not damaged by near-UV irradiation. The relationship between content of ergosterol and ATPase activity was examined using liposomes constructed with lipids extracted from the membrane. Maximum activity of ATPase was seen at 5\% ergosterol in liposomes; this activity was 2.5 times greater than that in liposomes without ergosterol. Activity of ATPase bound to liposomes with 5\% ergosterol was reduced after near-UV irradiation, while the activity remained unchanged in the case of the liposomes without ergosterol. Fluidity of the liposomes with 5\% ergosterol also decreased with increasing near-UV dosage. Dosage-response curves for reduction of ATPase activity and for decrease in fluidity were similar to that for photodecomposition of ergosterol. These results suggested that the reduction of ATPase activity in the membrane by near-UV irradiation was not caused by photochemical degradation of the primary structure of the ATPase molecule, but was attributable to conformational change resulting from an alteration in the higher-order structure of the membrane due to photochemical decomposition of ergosterol.

Keywords: near-UV, ergosterol, \textit{Saccharomyces cerevisiae}, ATPase, plasma membrane

INTRODUCTION

Near-UV irradiation reduces various functions of the plasma membrane and finally results in inactivation of the cell. However, the initial photochemical reactions and molecular mechanisms for these phenomena are little understood. We reported previously that ergosterol, which is one of the major components in the yeast plasma membrane, was photochemically decomposed (Arami \textit{et al.}, 1997) and that cell survival and membrane functions such as the permeability barrier and active transport were sigmoidally decreased by increasing doses of near-UV irradiation in \textit{Saccharomyces cerevisiae} (Arami \textit{et al.}, 1993).

Ergosterol plays an important role in the normal physiological function of the plasma membrane by regulating the delicate balance among other membrane components such as lipids and proteins. The fluid mosaic model (Singer & Nicolson, 1972) effectively shows interaction among these components and is significant in examining the transition of membrane-bound enzymes involved in membrane function. The physiological activity of membrane-bound proteins, which are in contact with other components, is sensitive to structural changes or physicochemical properties of other membrane components. Therefore, slight changes in membrane components may cause breakdown of the delicate balance of the plasma membrane and eventually lead to a reduction of membrane function.

Different components of the plasma membrane play important roles in membrane function. As regards physicochemical properties of the membrane, the acyl
chain type of phospholipids and the concentration of sterol compounds affect membrane permeability (Anderson & Thompson, 1992; Corvera et al., 1992; In't Veld et al., 1992), and asymmetry of phospholipids induces change in electrical potential of the membrane surface (Cerbon & Calderon, 1991). As to physiological function, peroxidation of lipids in the membrane reduces the activity of membrane-bound enzymes (Baba et al., 1981; Kukreja et al., 1988; Ohta et al., 1989; Scherer & Deamer, 1986) and cation transport (Marshanskii et al., 1983), and the length and degree of unsaturation in acyl chains of lipids influence active transport (Drissen et al., 1988; In't Veld et al., 1991, 1992). Moreover, molecular species of lipids also affect ATPase activity in the membrane (Corvera et al., 1992; Kasamo & Nouchi, 1987; Kasamo, 1990; Serrano et al., 1988). Like lipids, cholesterol also affects the activity of membrane-bound enzymes (George & McElhaney, 1992). Thus membrane functions are closely regulated by various membrane components.

In this study, we investigated the relationship between change in ATPase activity and decomposition of ergosterol by near-UV irradiation using both intact yeast plasma membranes and reconstructed liposomes.

**METHODS**

**Isolation of plasma membranes.** Saccharomyces cerevisiae IS66-4C α was grown in the dark at 26 °C in YPD medium (yeast extract 1%, bactopeptone 2% and glucose 2%, w/v) with continuous shaking at 125 r.p.m. for 20 h, at which time the culture was approaching stationary phase. After harvest, the yeast cells were washed twice with distilled water. Plasma membranes were isolated as described previously (Arami et al., 1997). The yeast cells were suspended in 0.1 M Tris/HCl buffer (pH 8.2) 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 washing the precipitate twice with the latter buffer, the plasma membrane fraction was collected using a sucrose density gradient at 100000 g, for 60 min at 4 °C. The layer containing the plasma membranes was identified by assay of Mg²⁺-ATPase, which was used as a marker enzyme. This layer was not contaminated by mitochondria since the ATPase activity was not inhibited in the presence of sodium azide.

**Lipid extraction and preparation of liposomes.** The total lipids from the plasma membrane fraction of yeast cells were extracted utilizing established procedures (Allen & Good, 1971) with slight modifications. The plasma membranes were suspended in chloroform/methanol (1:2, v/v) and stirred vigorously. After centrifugation at the suspension of 1000 g for 5 min, the residue was re-extracted with chloroform/methanol (1:1, v/v). A 0.5% NaCl solution was added to the chloroform layer with vigorous stirring, and the chloroform layer was concentrated by using a rotary vacuum evaporator. Total lipids extracted were fractionated using Silica Gel C-100 column chromatography (13 x 20 cm) in the order chloroform, acetone and methanol, separating neutral lipids containing ergosterol, glycolipids and phospholipids, respectively. Composition of the lipids separated was analysed by two-dimensional TLC. For TLC, Silica Gel 60 plates were activated by heating at 120 °C for 60 min and then cooled to room temperature. The plates were developed in the first dimension with chloroform/methanol/water (65:25:4, by vol.) and dried for 20 min under vacuum. The second solvent was chloroform/methanol/ammonia (65:35:5, by vol.). The lipids were identified by co-chromatography using lipid standards.

Reconstructed liposomes were produced as described by Darson et al. (1980). The ATPase solubilized from the plasma membrane of the yeast cells was incorporated deep into the liposomes by sonication with a bath sonicator. The ATPase was solubilized from the plasma membrane by established procedures (Davis & Hammes, 1989). Reconstructed liposomes were isolated by gel filtration with Sephadex G-100, using 10 mM Tris/HCl buffer (pH 7.4) containing 1 mM EDTA and 1 mM ATP.

**Irradiation procedure.** A fluorescent lamp (National FL20S.BL-B) was used as the light source (intensity 14 W m⁻², wavelength range 300–400 nm). The plasma membrane was suspended in deionized distilled water to a final concentration of 0.5 mg protein ml⁻¹ and was exposed to near-UV radiation with continuous stirring and ice cooling as previously described (Arami et al., 1997). When nitrogen gas or air was bubbled through during irradiation, the yeast cells were suspended in water degassed by sonication under reduced pressure. The flow rate of gases was 70 ml min⁻¹.

**Assay of enzyme activity.** ATPase activity was measured as described by Kasamo (1986) with slight modifications. The plasma membranes and reconstructed liposomes were suspended, to a final concentration of 1 and 0.2 mg protein ml⁻¹, respectively, in a reaction mixture containing 50 mM Tris/HCl (pH 7.8), 3 mM MgCl₂ and 2 mM ATP. After incubation at 30 °C for 10 min, the phosphate released from ATP was determined by established procedures (Fiske & Subbarow, 1925).

Activity of alkaline phosphatase was measured as described by Ito & Ito (1983). The plasma membranes were suspended, to a final concentration of 1.25 mg protein ml⁻¹, in a reaction mixture containing 0.2 M Tris/HCl (pH 8.5) and 10 mM p-nitrophenyl phosphate. After incubation at 30 °C for 30 min, 0.5 ml 1 M NaCO₃ was added to stop the reaction. The resultant p-nitrophenol was determined by measuring A₄₀⁰.

For assay of invertase, the plasma membranes were suspended, to a final concentration of 1 mg protein ml⁻¹, in 0.2 M acetate buffer (pH 5.2) containing 12.5 mM sucrose, and incubated at 30 °C for 5 h. The reaction was stopped by addition of 2 M NaOH and the pH of this mixture was adjusted to 7.0. After filtration through a cellulose acetate filter (pore size 0.45 μm), glucose produced was determined with mutarotase-GOD assay kit (Wako Pure Chemical Industries). Concentration of protein was determined using the Bio-Rad protein assay kit.

**Determination of membrane fluidity.** A pyrene derivative, which was a fluorescent probe for the lipid bilayer, was used for determination of membrane fluidity (Kitagawa & Takegaki, 1992). Pyrene docodecanoic acid was dissolved in THF (tetrahydrofuran) 1:1, v/v) to a final concentration of 0.35 mM and added to a suspension of reconstructed liposomes. After the suspension had been incubated at 30 °C for 20 min in the dark, it was excited at 340 nm and the fluorescence spectrum was measured at 350–550 nm.
Monomer and excimer peaks were detected at 397 and 464 nm, respectively. Fluorescence was determined by the ratio of fluorescence intensity of excimer to monomer. When the ratio of excimer to monomer was large, the fluorescence was high.

**Assay of the permeability barrier in liposomes.** The permeability barrier of liposomes was measured as previously described (Braganza et al., 1983) with modifications. Liposomes were prepared by using 10 mM Tris/HCl buffer (pH 7.0) containing phenol red (5 mg ml\(^{-1}\)) instead of distilled water in the method described above. After liposomes containing phenol red had been exposed to near-UV radiation at 25 °C, they were collected by centrifugation at 10,000 g for 30 min. Then 50 mM NaOH solution was added to the supernatant and the A\(_{550}\) was measured. When near-UV radiation induced damage in the permeability barrier of liposomes, phenol red leaked out, and the A\(_{550}\) increased with increased pH of the supernatant.

**Determination of ergosterol content.** Ergosterol content was determined as described previously (Arnezeder et al., 1989) with slight modifications. Samples exposed to near-UV were reflushed with 1 h in 5 ml of 90% (v/v) ethanol containing 7-dehydrocholesterol as an internal standard. After addition of 5 ml of 50% (v/v) ethanol, the sample was filtered through a cellulose acetate filter (pore size 0.45 μm). The filtrate was treated with a SEP-PAK C-18 cartridge (Millipore) and ergosterol was dissolved with 2-propanol. The solution containing ergosterol was analysed with an HPLC apparatus attached to a Nucleosil ODS column (4.5 × 150 mm; 5 μm particles; Chemco Scientific). 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 for absorbance at 280 nm.

**RESULTS**

Effect of near-UV irradiation on membrane bound enzymes

Near-UV irradiation caused changes in the activities of membrane-bound enzymes and in the ergosterol content of plasma membranes prepared from yeast cells (Fig. 1). Ergosterol content clearly decreased with increasing dosage, in parallel with reduction of activities of alkaline phosphatase and ATPase, which are well-known plasma-membrane-bound enzymes. The dosage–response curve for decrease in ergosterol content was very similar to that for reduction of enzyme activities. The activity of invertase in the plasma membrane preparations was reduced similarly to the above-mentioned enzymes by near-UV irradiation. Unlike membrane-bound ATPase, the activity of ATPase solubilized from the plasma membrane remained constant after exposure to near-UV, independent of dosage (data not shown). These results indicated that photodecomposition of ergosterol in the membrane might be closely related to reduction of activity of membrane-bound enzymes.

To examine the alteration in activity of membrane-bound enzymes by near-UV irradiation, we determined kinetic parameters for ATPase in the membrane exposed to various near-UV dosages. Maximum velocity of the ATPase was 17.2 μmol (mg protein)\(^{-1}\) min\(^{-1}\) at a dosage of 0 J ml\(^{-1}\); it decreased with increasing dosage, down to 15.6 μmol (mg protein)\(^{-1}\) min\(^{-1}\) at 2.5 J ml\(^{-1}\), and 10.0 μmol (mg protein)\(^{-1}\) min\(^{-1}\) at 5 J ml\(^{-1}\). On the other hand, the K\(_{m}\) value remained constant at approximately 23.6 mM before and after irradiation, indicating that near-UV radiation did not affect affinity between enzyme and substrate. It was supposed from this result that reduction of ATPase activity by near-UV was caused by alteration of the conformation of the enzyme, resulting from a change in the higher-order structure of the plasma membrane through the photodecomposition of ergosterol.

**Dependence on oxygen for reduction of ATPase activity and ergosterol content by near-UV**

We determined in our previous study that ergosterol photodecomposition with increasing dosage of near-UV was dependent on oxygen (Arami et al., 1997). Therefore, we examined whether reduction of membrane-bound ATPase activity depended on the presence of oxygen. Activity of ATPase in the plasma membrane exposed to near-UV with air bubbling clearly decreased with increasing dosage, in a similar manner to that shown in Fig. 1. However, ATPase activity remained constant at 1.8 μmol (mg protein)\(^{-1}\) min\(^{-1}\) with nitrogen gas bubbling. In the same way, the ergosterol content of the plasma membrane decreased with increasing near-UV dosage in the presence of oxygen but remained constant at 177 μg (mg protein)\(^{-1}\) in the absence of oxygen. These results indicated that the reduction in activity of membrane-bound ATPase accompanying photodecomposition of ergosterol was dependent on oxygen.
Effect of photodecomposition of ergosterol on ATPase activity in reconstructed liposomes

The effect of ergosterol on activity of membrane-bound enzymes was examined using liposomes prepared with ergosterol, lipids and ATPase from the plasma membrane. The normal plasma membrane is composed of many components such as proteins, lipids and various terpenoids containing photosensitizers, which can cause damage to other membrane components through generation of active oxygen. Therefore, we required exclusion of photosensitizers in these experiments. When the liposomes were exposed to near-UV radiation, photodecomposition of ergosterol and reduction of ATPase activity occurred simultaneously as in the case of the plasma membranes (Fig. 2). However, the activity of ATPase resolubilized from liposomes exposed at various dosages remained constant (Fig. 2b). These results indicated that reduction of membrane-bound ATPase activity was not directly induced by damage to the enzyme molecule, for example by destruction of peptide linkages or disulfide bonds. The reduction of ATPase activity might have occurred by alteration of the environment of the plasma membrane around the enzyme molecule.

It is well known that ergosterol plays a significant role in maintaining constant membrane function, for example membrane fluidity, the permeability barrier and the active transport system. We examined the relationship between concentration of ergosterol and ATPase activity using reconstructed liposomes. Maximum activity was seen at 5% ergosterol in liposomes (the same level as in the plasma membrane of intact cells); this activity was 2.5 times greater than for liposomes without ergosterol (Fig. 3). Liposomes prepared from lipids with and without 5% ergosterol were exposed to near-UV radiation. In the liposomes with ergosterol the higher activity of ATPase was markedly reduced with increasing dosage, but in the liposomes without ergosterol the lower activity remained constant (Fig. 4). These results indicated that ergosterol was essential to obtain higher activity of membrane-bound ATPase and that photodecomposition of ergosterol caused reduction of ATPase activity.
Reduction of membrane function by near-UV

**Effect of near-UV irradiation on membrane fluidity in liposomes**

It is well known that sterols and lipids are one of the regulatory factors for fluidity of the lipid bilayer. We examined the relationship between fluidity and ergosterol content by using liposomes prepared with lipids extracted from the plasma membrane of yeast cells (Fig. 5). Maximum fluidity was seen at 5% ergosterol content, and it decreased with decreasing ergosterol content; this indicated that ergosterol was necessary to obtain higher fluidity of the lipid bilayer.

Liposomes prepared from lipids with and without 5% ergosterol were exposed to near-UV radiation and the fluidity of the lipid bilayer was determined (Fig. 6). The fluidity decreased with increasing dosage in liposomes with ergosterol but did not change in liposomes without ergosterol. This result indicated that fluidity of the lipid bilayer was very closely related to photodecomposition of ergosterol. The dosage–response curve for decrease in fluidity was similar to the pattern of photodecomposition of ergosterol. Similarly, when the plasma membrane was exposed to near-UV radiation, the fluidity clearly decreased with increasing dosage as mentioned above.

In non-exposed liposomes with 3% ergosterol, fluidity was slightly higher than that of liposomes without ergosterol (Fig. 5). At a dosage of 5 J ml⁻¹, however, residual ergosterol in exposed liposomes was approximately 3% but relative fluidity was 0.48, which was lower than the fluidity of liposomes without ergosterol (Fig. 6). It is possible that membrane fluidity is lowered by photodegradation products of ergosterol in the lipid bilayer. We reported previously that the 5,7-diene structure of ergosterol in the plasma membrane was cleaved by near-UV radiation, indicating destruction of the sterol skeleton of ergosterol (Arami et al., 1997). It has been demonstrated on the basis of data using cholesterol that sterol skeletons act as relaxants for van der Waals contact between lipid molecules. Therefore, sterol cleavage would result in enhancing of this contact, which could lead to a decrease in membrane fluidity.

**Effect of near-UV irradiation on the permeability barrier**

The permeability barrier of yeast cells is known to be damaged by near-UV irradiation (Arami et al., 1993; Ito & Ito, 1983). We used liposomes to examine the relationship between disruption of the permeability barrier and photodecomposition of ergosterol. The leakage of phenol red from liposomes was examined as an indication of damage to the permeability barrier by near-UV radiation. Phenol red permeates the lipid bilayer in a temperature-dependent manner, and it does not permeate below 25 °C (Braganza et al., 1983). At this temperature the liquid phase state of the lipid bilayer is maintained. The phenol red in liposomes with ergosterol clearly leaked out with increasing near-UV dosage (Fig. 7). However, in the liposomes without ergosterol, leakage did not occur. In this experiment phenol red did not influence photodecomposition of ergosterol and was not decomposed by near-UV irradiation (data not shown).

In a series of experiments using liposomes, cholesterol was used in place of ergosterol since cholesterol is generally insensitive to near-UV radiation. Near-UV irradiation caused little or no reduction of ATPase activity, decrease in fluidity of lipid bilayer or destruction of permeability barrier in the liposomes with cholesterol.
Fig. 7. Leakage of phenol red from reconstructed liposomes after near-UV irradiation. Two types of liposomes were prepared, using glycolipids and phospholipids with (○) and without ergosterol (□) in 10 mM Tris/HCl buffer containing phenol red and were collected by gel filtration. After irradiation, the liposomes were removed and $A_{350}$ was measured to determine phenol red leakage. Each value represents the mean of results from three independent experiments (SE was within 5% of the mean).

**DISCUSSION**

Near-UV induces inactivation of S. cerevisiae, which, unlike the effects of far-UV, results from membrane damage rather than DNA damage (Ito & Ito, 1983). Pyrimidine dimers were not detected under our experimental conditions, indicating lesser significance of DNA damage in inactivation of yeast cells by near-UV radiation. It has been reported that the inactivation by near-UV radiation in S. cerevisiae was caused by damage to membrane function, for example the permeability barrier (Ito & Ito, 1983, 1984), and active transport (Arami et al., 1993). These facts indicate that the plasma membrane is a target for inactivation of the yeast cells by near-UV radiation.

It was described previously that dosage-response curves for inactivation of yeast cells by near-UV irradiation decreased sigmoidally with increasing dosage and were similar to that for decrease in active transport (Arami et al., 1993). Above a minimum level of ergosterol, yeast cells multiply normally (Leber et al., 1995). By analogy with our finding, it may also be possible to determine a range of ergosterol content at which a high level of active transport and cell multiplication are retained. However, the dosage–response curve for reduction of ATPase activity by near-UV irradiation showed no threshold and completely differed from those mentioned above. Activity of membrane-bound enzymes was very sensitive to decreased ergosterol content caused by near-UV. Therefore, it is suggested that photoinactivation of yeast cells occurs through accumulation of membrane damage such as reduction of activity of membrane-bound enzymes and active transport and that membrane damage can be taken as one of the fundamental factors in photoinactivation.

The function of the plasma membrane is maintained and regulated by a delicate balance of constituents in the membrane, as shown in the fluid mosaic model. Ergosterol constitutes approximately 5–7% of total lipid in the plasma membrane of the yeast cells, where it is available for regulation of fluidity and maintenance of the higher-order structure of the membrane, features which are necessary for stable maximal activity of membrane function. In practice, ergosterol was essential for stable construction of the membrane (Prasad, 1985). Needless to say, other lipids such as glycolipids and phospholipids play important roles in membrane function. Therefore, changes in membrane constituents would break down the delicate balance and eventually result in impairment of membrane function. By GLC analysis we determined that a decrease in content of unsaturated fatty acids actually occurred in several lipids of the membrane exposed to near-UV (data not shown), suggesting that peroxidative degradation of the lipids was induced by near-UV irradiation in a similar manner to that observed in previous studies using liposomal membranes (Bose et al., 1989, 1990). However, degradation of unsaturated fatty acids was slight compared with decomposition of ergosterol by near-UV irradiation. Similar results were also obtained in experiments using liposomes reconstructed with lipids extracted from membranes. Therefore, it is suggested that photodecomposition of ergosterol in the plasma membrane significantly affects the physical and chemical properties of the membrane, causing serious damage in membrane function such as lowering of the activity of membrane-bound enzymes.

Hu & Tappel (1992) reported that UV-A light had little or no effect on activities of either glyceraldehyde-3-phosphate dehydrogenase or alcohol dehydrogenase. In this study it was shown that ATPase activity did not change after near-UV irradiation of ATPase solubilized from the plasma membrane. In addition, the activities of ATPase isolated from plasma membranes or liposomes exposed to near-UV remained at normal levels irrespective of dosage. These results indicated clearly that near-UV irradiation had not damaged the primary structure of the enzyme molecule.

On the basis of these results, we suggest that reduction of ATPase activity in the plasma membrane by near-UV is not caused by the photochemical destruction of the primary structure of enzyme molecule, but is attributable to conformational changes in the membrane-spanning region of ATPase, caused by alteration in the higher-order structure of the plasma membrane due to photodecomposition of ergosterol. We propose that ergosterol plays an important role in photobiological responses related to membrane function of yeast cells.

**REFERENCES**


Reduction of membrane function by near-UV


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