Control of Singlet Oxygen-induced Oxidative Damage in *Escherichia coli*

Sun Yee Kim, Eun Ju Kim and Jeen-Woo Park*

Department of Biochemistry, College of Natural Sciences, Kyungpook National University, Taegu 702-701, Korea

Received 28 January 2002, Accepted 22 March 2002

Singlet oxygen ($^1\text{O}_2$) is a highly reactive form of molecular oxygen that may harm living systems by oxidizing critical cellular macromolecules. The *oxyR* gene product regulates the expression of the enzymes and proteins that are needed for cellular protection against oxidative stress. In this study, the role of *oxyR* in cellular defense against a singlet oxygen was investigated using *Escherichia coli oxyR* mutant strains. Upon exposure to methylene blue and visible light, which generates singlet oxygen, the *oxyR* overexpression mutant was much more resistant to singlet oxygen-mediated cellular damage when compared to the *oxyR* deletion mutant in regard to growth kinetics, viability and protein oxidation. Induction and inactivation of major antioxidant enzymes, such as superoxide dismutase and catalase, were observed after their exposure to a singlet oxygen generating system in both *oxyR* strains. However, the *oxyR* overexpression mutant maintained significantly higher activities of antioxidant enzymes than did the *oxyR* deletion mutant. These results suggest that the *oxyR* regulon plays an important protective role in singlet oxygen-mediated cellular damage, presumably through the protection of antioxidant enzymes.

Keywords: Singlet oxygen, *oxyR* regulon, Protein damage, Catalase, Superoxide dismutase

Introduction

Singlet molecular oxygen ($^1\text{O}_2$), an electronically-excited state of oxygen that is the result of the promotion of an electron to high-energy orbitals, is produced in mammalian cells under normal and pathophysiological conditions (Kanofsky, 1989). The photodynamic action of some drugs and pigments is also mediated through $^1\text{O}_2$ (Weishaupt *et al.*, 1976). $^1\text{O}_2$ is a highly reactive form of molecular oxygen that may harm living systems by oxidizing critical cellular macromolecules, including lipids, nucleic acids and proteins. It also promotes

deleterious processes, such as lipid peroxidation, membrane damage and cell death (Halliwell and Gutteridge, 1999).

The presence of a global regulatory mechanism for the coordinate expression of enzymes and proteins that are needed for cellular protection against oxidative stress is well documented (Christman *et al.*, 1985; Morgan *et al.*, 1986; Storz *et al.*, 1987). The induction of the oxidative response regulon is under positive control by the *oxyR* gene product, whose expression or activity is oxidant-inducible (Christman *et al.*, 1985). The *Escherichia coli* mutant *oxyR2* carries a dominant mutation that confers resistance to a variety of oxidizing agents. It also causes the overexpression of several enzymes that are involved in defenses against oxidative damage, whereas the deletions of *oxyR* are recessive and highly sensitive to oxidant (Christman *et al.*, 1985).

In the present study, the role of *oxyR* in cellular defense against a singlet oxygen-producing system was investigated using the *oxyR* overexpression mutant strain TA4110 and the *oxyR* deletion mutant strain TA4112. The two strains were expected to exhibit differences in regard to sensitivity to the toxic effects of singlet oxygen. To determine if such differences do indeed exist between the two types of cells, we examined growth kinetics, viability, modulation of activities of major antioxidant enzymes, and oxidative damage to protein upon exposure to methylene blue and visible light (MB/light), which generates singlet oxygen (Tabatabaie and Floyd, 1994). The data suggest that the *oxyR* regulon plays an important protective role in singlet oxygen-mediated cellular damage, presumably through the protection of antioxidant enzymes.

Materials and methods

Materials MB, 2,4-dinitrophenylhydrazine (DNPH), pyrogallol, and hydrogen peroxide were obtained from Sigma (St. Louis, USA). A Bio-Rad protein assay kit was obtained from Bio-Rad (Hercules, USA).

Bacterial strains and culture conditions The *E. coli* strains that harbored the *oxyR2* (TA4110, *oxyR*-constitutive) and the *oxyR Δ 3* (TA4112, *oxyR* deletion) genes were a kind gift of Dr. B. N. Ames (University of California, Berkeley). A minimal salt medium that

*To whom correspondence should be addressed.
Tel: 82-53-950-6352; Fax: 82-53-943-2762
E-mail: parkjw@knu.ac.kr

was supplemented with 0.5% glucose, 0.05% yeast extract and 10 mg/ml each arginine and methionine was used. It was designated as a GY medium. The rich medium, TSY, contained 3% trypticase soy broth plus 0.5% yeast extract. Overnight cultures that were grown in the GY medium at 37°C on a rotary shaker at 200 rpm were used to inoculate the fresh GY and TSY media. Exposure to MB/light began when the optical density (600 nm) reached 0.3–0.4. Bacterial cultures with various concentrations of MB were irradiated with white light from a 100 W tungsten lamp. Aliquots of the exposed cells were removed at time intervals and assayed for changes in absorbance at 600 nm. Viability was determined at various times by removing the samples and performing viable cell counts.

Protein carbonyl content The protein carbonyl content was determined spectrophotometrically using the described DNPH-labeling procedure (Levine *et al.*, 1994). The crude extract (~1 mg protein) was incubated with 0.4 ml 0.2% DNPH in 2 M HCl for 1 h at 37°C. The protein hydrazone derivatives were sequentially extracted with 10% (w/v) trichloroacetic acid, treated with ethanol/ethyl acetate, 1 : 1 (v/v), and re-extracted with 10% trichloroacetic acid. The resulting precipitate was dissolved in 6 M guanidine hydrochloride. The sample's spectrum difference, which was treated with DNPH in HCl, was examined versus a sample that was treated with HCl alone. Results are expressed as nmole of DNPH incorporated per mg of protein that was calculated from an absorptivity of 21.0 mM⁻¹cm⁻¹ at 360 nm for aliphatic hydrazones.

Activity of antioxidant enzymes The bacterial cells were collected at 10,000 × g for 10 min at 4°C and washed once with cold 50 mM potassium phosphate/0.1 mM EDTA at pH 7.8 before being resuspended in 1.0 ml of this buffer. Cell-free extracts were prepared by sonication. Cellular debris was removed by a 10-min centrifugation at 15,000 × g at 4°C. The supernatant was collected, and the protein levels were determined by the method of Bradford using bovine serum albumin as the standard. Catalase activity was measured in terms of the decomposition of hydrogen peroxide, which was followed directly by a decrease in absorbance at 240 nm (Beers and Sizer, 1952). SOD activity in the cell extracts was assayed spectrophotometrically using the pyrogallol assay as described (Kim *et al.*, 2001). One unit of activity is defined as the quantity of enzyme that reduces the superoxide-dependent color change by 50%.

In vitro inactivation Cell-free extracts of normally grown *E. coli* cells were exposed to 25 µM MB plus light and assayed for activities of SOD and catalase as described previously.

Statistical analysis A statistical analysis was performed by the student's *t*-test. A *p*-value of less than 0.05 was considered statistically significant.

Results

The growth kinetics of the TA4110 and TA4112 strains were compared following exposure to a singlet oxygen-generating system. Under aerobic conditions, the growth of both *oxyR*

Fig. 1. Growth of TA4110 (A) and TA4112 (B) upon exposure to MB/light. Exponentially growing bacteria were cultured at 37°C with shaking and exposed to MB and visible light. Increasing the optical densities of the cultures monitored cell growth. The result is representative of the three separate experiments. ◆, No addition; ■, 10 µM MB; ▲, 25 µM MB.

Fig. 2. Effect of MB/light on viability of *oxyR* cells. After the cells were exposed to 25 µM MB and various times of illumination, the viability was determined, as described in the text. The result is representative of the three separate experiments. ◆, TA4110; ■, TA4112.

strains were inhibited by MB/light in a concentration-dependent manner; however, the growth inhibition was more pronounced in TA4112 than in TA4110 (Fig. 1). Growth inhibition was also accompanied by the loss of cell viability. To determine the rate of viability loss, early exponential phase cells were incubated in liquid culture in the presence of MB with illumination, and subsequently plated to determine the colony-forming ability. The exponential phase was chosen since cells can become resistant to various forms of stress when they enter the stationary phase. The TA4110 cells were much more resistant to exposure of MB/light than TA4112 cells (Fig. 2). The TA4110 cells showed a 63% survival rate after exposure to 25 µM MB and illumination for 60 min compared with the TA4112 mutant cells, which showed a

Fig. 3. Protein carbonyl content of *oxyR* strains exposed to 25 μ M MB plus 2 h illumination. Protein carbonyls were measured in the cell-free extracts by the method of Levine and co-workers with the use of 2,4-dinitrophenylhydrazine (Levine *et al.*, 1994). Results are the mean $\pm$ S.D. of the three determinations. * $p < 0.01$ compared to untreated cells.

32% survival rate. Exposure of *oxyR* strains to MB in the absence of illumination, or by illumination alone, did not modify the viability of cells. The strain-deleted *oxyR* was killed to a greater extent upon exposure to MB/light, indicating that *oxyR* may be involved in protecting the cells from singlet oxygen-mediated cellular damage.

Protein oxidation that is mediated by the reactive oxygen species is accompanied by the conversion of proline, lysine, arginine and histidine residues into carbonyl derivatives (Amici *et al.*, 1989), therefore providing a convenient assay of oxidative modification. The carbonyl content of the protein can be measured using a phenylhydrazine formation reaction. We measured the carbonyl levels for protein oxidation 2 h after cellular exposure to 25 μ M MB/light in order to determine whether the *oxyR* expression decreased sensitivity to protein damage. As shown in Fig. 3, protein oxidation was significantly increased in both strains of the incubation with MB/light. However, the TA4112 cells exhibited significantly higher levels of carbonyl groups compared to TA4110.

To determine whether the expression of the *oxyR* gene induces concomitant alteration in the activity of major antioxidant enzymes, the modulation of the activities of catalase and SOD upon exposure to MB/light was examined. When the two *oxyR* strains were exposed to MB/light, the catalase activity increased; however, significantly higher activity was observed in TA4110 compared to TA4112 (Fig. 4). The activity of SOD similarly increased in the two *oxyR* strains upon exposure to MB/light, although slightly lower activity was observed in TA4112. These data do not, however, establish whether this increase was due to de novo synthesis or to the activation of pre-existing proteins. The effect of MB/light on the activities of antioxidant enzymes was examined in cell-free extracts that lacked induction capabilities. Fig. 5 demonstrates that antioxidant enzymes in the two *oxyR* strains were inactivated upon exposure to MB/light in a time-dependent manner, although the rate of inactivation was slower in TA 4110 cells. These results indicate that

Fig. 4. Effect of MB (25 μ M)/light (2 h) on the activities of catalase (A) and SOD (B) in *oxyR* strains. Results are the mean $\pm$ S.D. of the three determinations. * $p < 0.01$ compared to untreated cells.

Fig. 5. Inactivation of catalase (A) and SOD (B) in cell-free extracts from *oxyR* strains upon exposure to 25 μ M MB plus visible light. Activity of untreated cell-free extract is expressed as 100%. Results represent the means of the three determinations. $\blacklozenge$, TA4110; $\blacksquare$, TA4112.

antioxidant enzymes are susceptible to the damage that is caused by MB/light. This observation suggests that singlet oxygen, besides inducing the synthesis of catalase and superoxide dismutase, could also cause inactivation of these enzymes.

Discussion

We know that $^1\text{O}_2$ can be generated in cells, such as under conditions of oxidative stress (Sies, 1986), decomposition of lipid peroxides, or by spontaneous dismutation of superoxide (Krinsky, 1974; Foote, 1977). In addition, both naturally occurring compounds such as riboflavin (Joshi, 1985) and many xenobiotics, such as psoralene (de Mol *et al.*, 1981), porphyrins (Weishaupt *et al.*, 1976) and tetracyclins (Hasan and Khan, 1986), can generate $^1\text{O}_2$ inside cells when irradiated by visible light. Singlet oxygen is usually generated *in vitro* by photosensitization reactions. Irradiation of the sensitizer dye, such as MB or RB, mediates photoreactions through an

excited triplet state. It acts either by hydrogen atom or electron transfer reactions (type I) or by transferring the excited energy, forming singlet oxygen, which then reacts with the target molecules (type II) (Foote, 1977).

Light induced a few diseases, including erythropoietic protoporphyria, pellagra and cataractogenesis that have been attributed in part to the toxicity of ${}^1\text{O}_2$ (Mathews-Roth, 1986; Egorov *et al.*, 1987). On the other hand, singlet oxygen is the primary active species in a novel cancer treatment modality known as photodynamic therapy (Henderson and Dougherty, 1992).

This study demonstrated that the oxidative defense regulon *oxyR* plays an important role in protection from toxicity that is induced by singlet oxygen. We have shown that the *oxyR* deletion mutant is hypersensitive to damage that is caused by singlet oxygen. Cells that lack the expression of the *oxyR* gene show a significant decrease in cell viability (Fig. 2) and increase in oxidative damage to the protein (Fig. 3). This finding emphasizes the essential role of the *oxyR* regulon in mitigating such damage.

Although antioxidant enzymes play a major role in the cellular defense mechanism, they are susceptible to inactivation by reactive oxygen species. The oxidative processes result in the loss of key antioxidant enzymes (Kono and Fridovich, 1982; Tabatabaie and Floyd, 1994), which may exacerbate oxidative stress-mediated cytotoxicity. However, it is also possible that prokaryotes and eukaryotes compensate for the inactivation of antioxidant enzymes by the enhanced expression of SOD, catalase, and other antioxidant enzymes. Induction of antioxidant proteins in response to oxidative stress in *E. coli* and *Salmonella typhimurium*, as well as mammalian cells, is well known (Christman *et al.*, 1985; Greenberg *et al.*, 1990). Our results show that catalase and SOD activities were induced, and that the activity of catalase in the *oxyR* overexpression mutant is significantly higher than that in the *oxyR* deletion mutant. In contrast, the activity of SOD in TA4110 was higher to a lesser extent in the absence or presence of MB/light compared to TA4112 (Fig. 4). The SOD activity is not regulated by *oxyR* (Demple and Halbrook, 1983; Bowen and Hassan, 1988), although the *oxyR*-overexpressed mutant constitutively produces a high level of catalase, and presumably other antioxidant proteins. The *soxR* regulon positively controls ~15 genes in *E. coli* including SOD (Greenberg *et al.*, 1990). Singlet oxygen may play a role directly or indirectly in the *soxR* regulon-mediated induction of SOD. When cell-free extracts from both *oxyR* strains were exposed to singlet oxygen, the catalase and SOD in TA4112 were more susceptible to oxidative inactivation (Fig. 5). These *in vivo* and *in vitro* results indicate that a higher level of some antioxidant enzymes may protect catalase and SOD from damage that is mediated by singlet oxygen. It may eventually

prevent cells from progressing to the pro-oxidant state. This protection may be a mechanism that increases survival and decreases protein damage in the *oxyR* overexpression mutant.

In conclusion, results of this study indicate that a deficiency of *oxyR* increased the sensitivity of *E. coli* to the singlet oxygen-generating system, such as MB/light. The *oxyR* regulon also provides protection against the oxidative inactivation of SOD and catalase through the induction of antioxidant enzymes. These results provide support for the role of *oxyR* as an important regulator to protect cells against singlet oxygen-mediated oxidative stress.

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