Oxidative stress response in yeast: effect of glutathione on adaptation to hydrogen peroxide stress in *Saccharomyces cerevisiae*

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Abstract Role of intracellular glutathione in the response of *Saccharomyces cerevisiae* to H_2O_2 was investigated. Depletion of cellular glutathione or inhibition of γ-glutamylcysteine synthetase (GSH-I) enhanced the sensitivity to H_2O_2 and suppressed the adaptation to H_2O_2. A mutant deficient in GSH-I also showed the hypersensitivity and could not adapt to H_2O_2. Incubation of the cell with amino acids constituting glutathione (L-Glu, L-Cys, Gly) increased the intracellular glutathione content, and subsequently the cell acquired resistance against H_2O_2. These results strongly suggest that intracellular glutathione plays an important role in the adaptive response in *S. cerevisiae* to oxidative damage.

Key words: Glutathione; L-Buthionine sulfoximine; 1-Chloro-2,4-dinitrobenzene; Hydrogen peroxide; *Saccharomyces cerevisiae*

1. Introduction

All aerobic organisms use molecular oxygen ($^3O_2$) for respiration or oxidation of nutrients to obtain energy efficiently. During the reduction of molecular oxygen to water through acceptance of four electrons, active oxygen species such as superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\bullet}$) are generated. These active oxygen species attack almost all cell components, DNA, protein and lipid membrane. Active oxygen species are known to be causative of degenerative diseases such as cancer [1,2]. Against such active oxygens, cells have some defensive mechanisms including superoxide dismutase, catalase, several peroxidases and antioxidants such as ascorbate, tocopherol, uric acid, β-carotene and glutathione.

Glutathione is widely found in microorganisms, plants and animals. It has various functions in the defense against oxidative stress and xenobiotic toxicity [3–6]. In animals, H_2O_2 generated through respiration is scavenged in mitochondria by glutathione peroxidase (GPx). Thus glutathione has an essential function in mitochondria under normal physiological condition in animals, and glutathione deficiency leads to several diseases by damage of mitochondria [7,8].

Much previous work about oxidative stress responses of *S. cerevisiae* has focused on stress-inducible proteins and membrane lipid unsaturation, and has shown that there are distinct inducible stress responses to both peroxides and superoxides [9–12]. Davies et al. have reported that adaptation to H_2O_2 requires protein synthesis and expression of at least 21 proteins increased following H_2O_2 adaptation [13]. On the other hand, Wu and Moye-Rowley reported that expression of *GSH1* gene encoding γ-glutamylcysteine synthetase is regulated by yAP-1, a transcriptional regulator of the oxidative stress response in *S. cerevisiae* [14]. γ-Glutamylcysteine synthetase is a key enzyme for glutathione biosynthesis. However, information about the role of glutathione on the stress response in *S. cerevisiae* is largely lacking. In this study we examined the function of intracellular glutathione on H_2O_2 stress response in yeast.

2. Materials and methods

2.1. Yeast strains and media

S. cerevisiae S288C (*MAT***a** *SUC2 mal mel gal2 CUP1*) was obtained from Yeast Genetic Stock Center, University of California, Berkeley, USA. YNN27 (*MATα trp1 ura3*) and YH-1 (*MATα trp1 ura3 gsh1*) were kindly donated by Dr. Y. Ohtake, Asahi Breweries Ltd. Cells of S288C were cultured in YPD medium (2% glucose, 2% peptone, 1% yeast extracts) at 28°C with reciprocal shaking. YNN27 and YH-1 were cultured in SD medium (2% glucose, 0.67% yeast nitrogen base, 20 μg/ml of L-tryptophan and uracil). Log phase cells were harvested at the optical density of the culture at 610 nm (OD_{610}) of 0.1. Stationary phase cells were harvested after cultivation for 72 h.

2.2. H_2O_2 treatment

The condition for treatment of the cells was essentially the same as described by Flattery-O'brien et al. [11]. Cells were harvested and resuspended in 100 mM potassium phosphate buffer (pH 7.4) to obtain the initial $OD_{610} = 0.1$. To observe the sensitivity of yeast to H_2O_2, various concentrations of H_2O_2 were added to 5 ml samples, and cell survival was monitored by taking samples at 15 min intervals, diluting by the same buffer and plating aliquots on YPD plates. For adaptation experiments, cells were resuspended in fresh YPD medium containing a sublethal concentration of H_2O_2 (0.2 mM) and incubated with shaking at 28°C for 1 h. Pretreated cells were harvested and resuspended in 100 mM potassium phosphate buffer (pH. 7.4), and challenged to the lethal concentration of H_2O_2 (2 mM). SD medium was used instead of YPD for YNN27 and YH-1, and stress conditions were as follows: first stress 0.01 mM for 1 h, second stress 0.1 mM.

2.3. Assay of glutathione-producing activity

Assays of glutathione-producing activity and extraction of glutathione from cells were carried out essentially the same as those described by Murata et al. [15]. Cells were incubated in a mixture containing 0.5 M glucose, 0.01 M $MgCl_2$, 0.02 M L-glutamate, 0.02 M L-cysteine, 0.02 M glycine and 0.1 M potassium phosphate buffer (pH 7.4) at 28°C for 1 h with shaking. After the incubation, cells were collected by centrifugation and washed twice with distilled water. Glutathione in the cells was then extracted and determined by the method of Tietze [16]. Glutathione-producing activity was expressed as the amount of glutathione formed in 1 h per g (as wet weight) of the cells.

2.4. Enzyme activities

Cells were disrupted by vortexing with glass beads in 100 mM potassium phosphate buffer (pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride and 1 μg/ml pepstatin A. Glutathione reductase activity was measured according to the method of Racker [17]. Glucose-6-phosphate dehydrogenase activity was measured by the method of Kornberg and Horecker [18]. Protein was measured by the method of Lowry et al. [19].

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2.5. Depletion of intracellular glutathione

To decrease intracellular glutathione content, cells were incubated in YPD medium containing 0.5 mM 1-chloro-2,4-dinitrobenzene (CDNB) for 1 h. To increase glutathione content, cells were incubated in the mixture containing L-glutamate, L-cysteine and glycine as described above for 1 h. To inhibit GSH-I activity, cells were incubated in YPD medium containing 1 mM L-buthionine sulfoximine (BSO).

3. Results

3.1. Effect of glutathione content on the sensitivity to H_2O_2

It has been reported that if the cells of microorganisms enter into the stationary phase, the cells acquire resistance against several environmental stresses such as heat, oxidative damage and osmotic stress [20–22]. We also confirmed that cells of *S. cerevisiae* S288C in stationary phase showed higher resistance to H_2O_2 (data not shown), which was previously reported by Steels et al. [23]. It was thought to be due to the increased expression of some genes encoding stress shock proteins. In the meanwhile, as shown in Table 1 , intracellular glutathione content of cells in the stationary phase was approximately 3-fold higher than that in log phase cells. Therefore, we speculated that intracellular glutathione level may also be one of the factors that determine the resistance against several stresses.

Intracellular glutathione (GSH + GSSG) can be depleted by treatment with CDNB. Approximately 50% of intracellular glutathione was abolished by treatment of the cells with 0.5 mM CDNB for 1 h (Table 1). Fig. 1 shows the effect of CDNB on the sensitivity of the cells against H_2O_2. The cells pretreated by CDNB became hypersensitive to H_2O_2.

Biosynthesis of glutathione is catalyzed by two enzymes, i.e., GSH-I (γ-glutamylcysteine synthetase) and GSH-II (glutathione synthetase). BSO is a potent inhibitor of GSH-I, and the glutathione content of the cells treated by BSO (1 mM for 1 h) was also decreased compared with that of the control cells (Table 1). Pretreatment of the cells by BSO also made the cells sensitive to H_2O_2 (Fig. 1).

Glutathione is a tripeptide consisting of L-glutamate, L-cysteine and glycine. Incubation of the cells with these amino acids increased the intracellular glutathione level (Table 1) [15], and consequently the cells showed a higher resistance against H_2O_2 (Fig. 1).

To confirm the effect of glutathione content on stress response, the sensitivity of glutathione biosynthesis-deficient mutant YH-1 to H_2O_2 was investigated. YH-1 is lacking the GSH-I activity and does not contain glutathione in the cells [24]. As shown in Fig. 2, YH-1 was much more sensitive to H_2O_2 than YNN27, which is a parent strain of YH-1. YNN27 could survive in 100 mM potassium phosphate buffer (pH 7.4) containing 0.5 mM H_2O_2 for 1 h (Fig. 2A), whereas 99% of the mutant cells were killed under the same conditions (Fig. 2B).

Table 1
Intracellular glutathione content under several conditions

	Glutathione content (μmol/g cell)
Log phase cell	2.22 ± 0.04
Stationary phase cell	6.89 ± 0.07
CDNB treatment[a]	1.23 ± 0.08
BSO treatment[b]	1.98 ± 0.07
Incubation with amino acids[c]	18.47 ± 0.88
0.2 mM H_2O_2	2.47 ± 0.03

Values are shown as the average of four independent experiments.
[a] Cells were treated by 0.5 mM CDNB for 1 h.
[b] Cells were treated by 1 mM BSO for 1 h.
[c] Cells were incubated with 0.02 M L-glutamate, L-cysteine and glycine for 1 h.

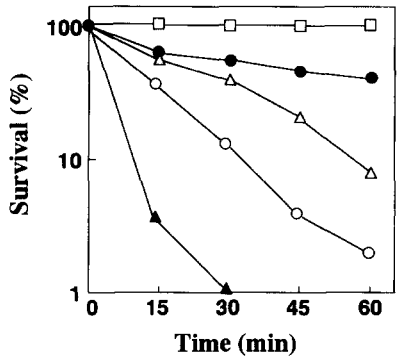

Fig. 1. Effect of intracellular glutathione content on sensitivity to H_2O_2. Cells of S288C in log phase were treated with 0.5 mM CDNB (▲), 1 mM BSO (○) for 1 h in YPD medium at 28°C, or incubated in a mixture containing 0.02 M L-glutamate, L-cysteine and glycine for 1 h at 28°C (●), respectively, and then challenged to H_2O_2 (2 mM). △, cells were directly challenged to H_2O_2 (2 mM) without pretreatment. □, cells were not treated with any chemicals (as control).

3.2. Effect of glutathione content on the adaptation to H_2O_2

It has been known that pretreatment of the cells by sublethal concentration of H_2O_2 renders the cell resistance against subsequent treatment by lethal concentration of H_2O_2. The phenomenon was observed in both prokaryotic and eukaryotic microorganisms, including *S. cerevisiae* [9,11–13], and termed as adaptation.

We also confirmed that adaptation was induced by pretreatment of *S. cerevisiae* S288C by sublethal concentration (0.2 mM) of H_2O_2 (Fig. 3A). The adaptation was suppressed if the cells were treated with 0.2 mM H_2O_2 in the presence of 0.5 mM CDNB (Fig. 3A). Partial inhibition was also observed if the pretreatment by H_2O_2 was carried out with 1 mM BSO (Fig. 3A). Furthermore, adaptation was not observed in the case of glutathione biosynthesis-deficient mutant YH-1 (Fig. 3B). These results suggest that intracellular content of glutathione affects the adaptation to oxidative stress caused by H_2O_2.

3.3. Change of glutathione producing and recycling activities

Activities of enzymes involved in biosynthesis and recycling of glutathione were measured. Glutathione producing activity slightly (approximately 10%) increased by treatment of the cells with 0.2 mM H_2O_2 for 1 h. Glutathione disulfide (GSSG) is reduced to a reduced form of glutathione (GSH) by glutathione reductase (GR) in the presence of NADPH. $NADP^+$ thus formed is reduced to NADPH by action of glucose-6-phasphate dehydrogenase (G6PDH). Both GR and G6PDH are involved in the glutathione recycling system. As shown in Table 2, both enzyme activities increased approximately 1.5-fold after the treatment with 0.2 mM H_2O_2 for 1 h. Total intracellular glutathione content slightly increased by the same treatment (Table 1). These results suggest that *S. cerevisiae* cells seem to adapt to H_2O_2 stress by increasing both glutathione content and glutathione recycling activities.

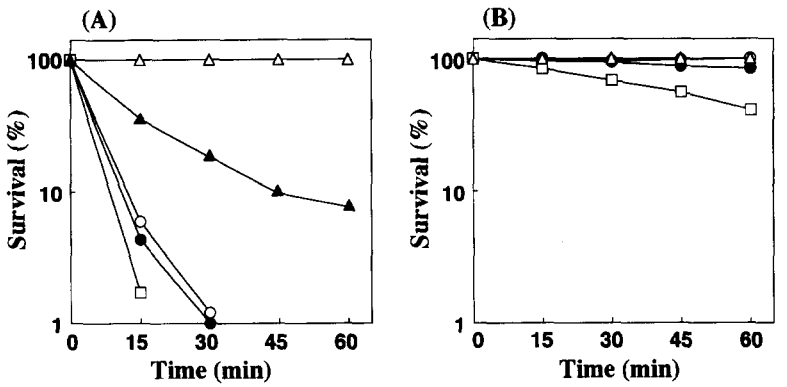

Fig. 2. Sensitivity of glutathione-deficient mutant YH-1 (A) and its parental strain YNN27 (B) to H_2O_2. Cells were cultured in SD medium to log phase and treated with various concentrations of H_2O_2 for 1 h. Samples were diluted and plated on YPD agar plates to monitor the cell viability. H_2O_2 concentrations and symbols were: 0 mM (△); 0.1 mM (▲); 0.2 mM (○); 0.5 mM (●); 1 mM (□).

4. Discussion

In the mammalian system, Meister and Anderson proposed that glutathione protects the cells from oxidative damage [5]. Contrasting to this, Greenberg and Demple reported that *gshA*::*Tn*10km mutant of *Escherichia coli* K-12 has normal resistance to H_2O_2 and cumene hydroperoxide [25]. The *gshA* gene encodes γ-glutamylcysteine synthetase (GSH-I). GSH-I is a rate-limiting enzyme in the biosynthesis of glutathione, and intracellular glutathione content in the *gshA*::*Tn*10km mutant decreased to <0.4% compared to that of wild type strain. Therefore, Greenberg and Demple concluded that intracellular glutathione did not protect *E. coli* from oxidative damage.

On the other hand, we demonstrated that intracellular glutathione played an important role in the stress response to H_2O_2 in *S. cerevisiae* using glutathione depleting agents and a glutathione-deficient mutant. Sensitivity to H_2O_2 increased when *S. cerevisiae* cells were treated by glutathione depleting agents such as CDNB and BSO, whereas incubation of the cells with amino acids (L-Glu, L-Cys, Gly) which constitute glutathione increased the intracellular glutathione content, and subsequently the cells acquired resistance to H_2O_2. *S. cerevisiae* YH-1, which is a glutathione-deficient mutant, was hypersensitive to H_2O_2 compared with its parental strain, and did not show the adaptation. Cellular glutathione was not detected in the mutant deficient in *gsh1* gene in *S. cerevisiae* [24]. Therefore, *S. cerevisiae* may have a similar mechanism as observed in mammalian cells to protect the cells against the oxidative damages.

Many studies have been reported on the relationship between glutathione and stress response in animal cells [7,8,26–29]. In mammalian cells, glutathione peroxidase (GPx) has a primary function to scavenge H_2O_2. H_2O_2 is reduced to H_2O by GPx. Glutathione in reduced form (GSH) is an electron donor for GPx reaction, and oxidized form of glutathione (GSSG) is reduced to GSH by glutathione reductase (GR) in the presence of NADPH. $NADP^+$ is reduced to NADPH by glucose-6-phosphate dehydrogenase (G6PDH). In *E. coli* and *Salmonella typhimurium*, *gorA* gene, which encodes GR, is involved in the H_2O_2-inducible *oxyR* regulon [30]. As shown in Table 2, treatment of *S. cerevisiae* cells with sublethal concentration of H_2O_2 induced both GR and G6PDH. Glutathione synthesizing activity was also slightly increased by H_2O_2 treatment, and BSO which is a potent inhibitor of GSH-I suppressed the adaptation. Therefore, de novo synthesis of glutathione and recycling of glutathione are likely to be involved in the adaptation system in *S. cerevisiae*.

We have previously reported that a yeast *Hansenula mrakii*

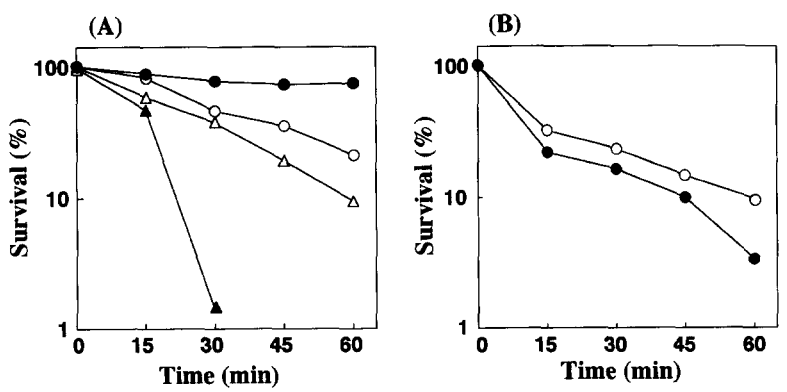

Fig. 3. Effect of intracellular glutathione content on adaptation. (A) Cells of S288C in log phase were pretreated with 0.2 mM H_2O_2 (●); 0.2 mM H_2O_2 and 1 mM BSO (○); 0.2 mM H_2O_2 and 0.5 mM CDNB (▲) for 1 h, respectively, and then challenged to H_2O_2 (2 mM). △, cells were directly challenged to H_2O_2 (2 mM) without pretreatment. (B) Cells of YH-1 in log phase were pretreated with 0.01 mM H_2O_2 (●) for 1 h, and then challenged to H_2O_2 (0.1 mM). ○, cells were directly challenged to H_2O_2 (0.1 mM) without pretreatment.

Table 2
Change of enzyme activities by H_2O_2 treatment

Treatment with H_2O_2	GSH-I+GSH-II[a]	GR[b]	G6PD[c]
Not treated	16.25 ± 0.88 (100)	28.7 ± 0.9 (100)	461 ± 54 (100)
0.2 mM, 1 h	17.81 ± 2.03 (110 ± 12)	41.7 ± 4.3 (145 ± 15)	607 ± 131 (151 ± 28)

Values are shown as the average of four independent experiments.
[a] μmol glutathione formed/h/g(wet-wt)-cells
[b] mU/mg protein; 1 unit (U) of the activity was defined as the amount of enzyme reducing 1.0 μmol of GSSG per min at 25°C.
[c] mU/mg protein; 1 unit (U) of the activity was defined as the amount of enzyme oxidizing 1.0 μmol of D-glucose 6-phosphate per min at 25°C.
Parentheses show the relative activity of each enzyme. The activities of non-treated cells are relatively taken as 100%.

has a membrane-bound GPx which was induced by lipid hydroperoxide [31,32]. In such a case, G6PDH was also induced to supply NADPH [33]. However, it is still controversial whether *S. cerevisiae* has GPx or not. If we assume that *S. cerevisiae* has a GPx, it is entirely reasonable that *S. cerevisiae* increases intracellular glutathione content as an electron donor for GPx reaction and adapts to H_2O_2 stress in analogy with mammalian cells. In the case of *S. cerevisiae* does not have a GPx, we can still give some explanations for the increase of intracellular glutathione content to protect the cell against oxidative stress. For example, glutathione can directly scavenge the reactive oxygen species. It is well known that glutathione is a scavenger of $HO^{\bullet}$ and singlet oxygen ($^1O_2$) [2,3,34]. $HO^{\bullet}$ and $O_2^{\bullet -}$ are formed by Fenton reaction from H_2O_2 in the presence of Fe(II). Alternatively, glutathione can reactivate disulfide bonds (–SS–) of some proteins to generate sulfhydryl (–SH) groups, which may be involved in essential sites of such proteins [3,35]. It is likely that glutathione contributes to sustentation the functions of such proteins. Therefore, the increase of intracellular glutathione may be one of the adaptations to H_2O_2 stress in *S. cerevisiae*.

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