Role of glutathione in heat-shock-induced cell death of *Saccharomyces cerevisiae*

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Previously we reported that expression of *GSH1* (γ-glutamylcysteine synthetase) and *GSH2* (glutathione synthetase) of the yeast *Saccharomyces cerevisiae* was increased by heat-shock stress in a Yap1p-dependent fashion and consequently intracellular glutathione content was increased [Sugiyama, Izawa and Inoue (2000) J. Biol. Chem. **275**, 15535–15540]. In the present study, we discuss the physiological role of glutathione in the heat-shock stress response in this yeast. Both *gsh1* and *gsh2* mutants could acquire thermotolerance by mild heat-shock stress and induction of Hsp104p in both mutants was normal; however, mutant cells died faster by heat shock than their parental wild-type strain. After pretreatment at a sublethal temperature, the number of respiration-deficient mutants increased in a *gsh1* mutant strain in the early stages of exposure to a lethal temperature, although this increase was partially suppressed by the addition of glutathione. These results lead us to suspect that an increase of glutathione synthesis during heat-shock stress is to protect mitochondrial DNA from oxidative damage. To investigate the correlation between mitochondrial DNA damage and glutathione, mitochondrial Mn-superoxide dismutase (the *SOD2* gene product) was disrupted. As a result, the rate of generation of respiration-deficient mutants of a *sod2*Δ strain was higher than that of the isogenic wild-type strain and treatment of the *sod2*Δ mutant with buthionine sulphoximine, an inhibitor of glutathione synthesis, inhibited cell growth. These results suggest that glutathione synthesis is induced by heat shock to protect the mitochondrial DNA from oxidative damage that may lead to cell death.

Key words: mitochondrial DNA, reactive oxygen species, respiration deficiency, Yap1p.

INTRODUCTION

A sudden increase in environmental temperature induces production of several heat-shock proteins (HSPs). HSPs are believed to be necessary for cells to acquire thermotolerance. Many HSPs function as molecular chaperones in protein folding. It is known that chaperones have ATPase activity [1]. Aerobic organisms can produce ATP through oxygen respiration much more efficiently than anaerobic organisms that produce it by glycolysis. In eukaryotes, the electron-transport chain (respiratory chain) exists in mitochondria; therefore, the organelle is a major source for reactive oxygen species. In our previous paper [2], we reported that the oxygen respiration rate of the yeast *Saccharomyces cerevisiae* was increased by heat-shock stress, which resulted in increased intracellular oxidation levels. Therefore, heat-shock stress may be, in some part, equivalent to oxidative stress.

During reduction of molecular oxygen to H_2O, several reactive oxygen species, such as superoxide radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO^{\bullet}$), are formed. The $O_2^{\bullet -}$ is disproportionated to O_2 and H_2O_2 by superoxide dismutase (SOD), and the H_2O_2 formed is decomposed to H_2O and O_2 by catalase or reduced to H_2O by several peroxidases. In addition to so-called *HSP* genes, several genes involved in antioxidant systems have been known to be induced by heat shock in *S. cerevisiae*, such as *CTT1* [3] and *TSA1* [4]. The *CTT1* gene encodes cytosolic catalase [5]. The null mutant of *CTT1* is sensitive to heat shock, while overexpression of the gene enhances resistance to lethal heat-shock stress [6]. The *TSA1* gene product is a thioredoxin peroxidase (TPx) [7–9]. Lee and Park [4] reported that expression of *TSA1* was induced by heat shock and disruption of the gene increased susceptibility to heat stress. We found that expression of *GSH1* and *GSH2* in *S. cerevisiae* was induced by heat shock and consequently intracellular GSH content was increased [2].

GSH is a non-proteinous thiol compound abundant in almost all aerobic organisms, and is synthesized in two sequential reactions catalysed by γ-glutamylcysteine synthetase (the *GSH1* gene product) and glutathione synthetase (*GSH2* gene product) in the presence of ATP. GSH has many physiological functions in cells [10,11]. One of its most important functions is to act as an antioxidant. GSH can react directly with $HO^{\bullet}$ to reduce it to H_2O [12]. Besides this, GSH can serve as an electron donor for glutathione peroxidase, which catalyses reduction of H_2O_2 and lipid hydroperoxide to H_2O and the corresponding alcohol [13,14]. We have been studying the oxidative-stress response in yeasts and have reported that GSH is one of the important factors that determines resistance against oxidative stress [15–18].

In addition to its role as an antioxidant, GSH has several physiological functions, such as detoxification of various cytotoxic compounds, acting as a co-factor for enzymes, protection of proteins' SH groups, transportation of amino acids (constituting the γ-glutamyl cycle) [11,19]. For instance, if cells are invaded by xenobiotics, glutathione-S-conjugates are formed by glutathione S-transferase and the conjugates or their degraded compounds are exported from the cytoplasm by some transporters. In *S. cerevisiae*, two glutathione S-transferase genes (*GTT1* and *GTT2*) have been identified [20] and glutathione-S-conjugates are transported into the vacuole by the *YCF1* gene

Abbreviations used: HSP, heat-shock protein; SOD, superoxide dismutase; RD, respiration-deficient; mtDNA, mitochondrial DNA; BSO, buthionine sulphoximine; CDNB, 1-chloro-2,4-dinitrobenzene; TRX, thioredoxin; TPx, thioredoxin peroxidase; γ-GC, γ-L-glutamyl-L-cysteine.

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product, which is an ATP-binding-cassette transporter on the vacuolar membrane [21]. Choi et al. [20] reported that deletion mutants of *GTT1* and/or *GTT2* exhibited increased susceptibility to heat shock and limited growth at 39 °C. Taken together, GSH seems to play some physiological roles in the heat-shock stress response in *S. cerevisiae*.

In this paper, we demonstrate that one of the physiological roles of induction of GSH synthesis by heat shock in yeast is to protect the mitochondrial DNA (mtDNA) from oxidative damage and that GSH can partially suppress cell death induced by heat shock.

MATERIALS AND METHODS

Strains

S. cerevisiae YNN27 (*MATα trp1-289 ura3-32 gal2*), YH1 (*MATα trp1-289 ura3-32 gal2 gsh1*) and YL1 (*MATα trp1-289 ura3-32 gal2 gsh2*) were provided by Dr Y. Ohtake (Asahi Breweries, Ibaraki, Japan) [22]. *S. cerevisiae* YPH250 (*MAT***a** *trp1-Δ1 his3-Δ200 leu2-Δ1 lys2-801 ade2-101 ura3-52*) and S288C (*MATα SUC2 mal mel gal2 CUP1*) were obtained from the Yeast Genetic Stock Center, University of California at Berkeley, CA, U.S.A.

Gene disruption

The open reading frame of *SOD2* was cloned by PCR using the following two primers: 5′-CATATGTCGACGCCACCATCC-TATC-3′ and 5′-GATCTTGCCAGCATCGAATCTTCTGG-ATGC-3′. The genomic DNA of *S. cerevisiae* S288C was used as a template. The PCR product (1247 bp) was treated with Klenow, and cloned into the *Hin*cII site of pUC19 to yield the plasmid pUC19+*SOD2*. To construct the *sod2*-disruption plasmid, pUC19+*SOD2* was digested with *Nhe*I, treated with Klenow, and then the *ADE2* gene was inserted to yield the plasmid pUC19-*sod2*::*ADE2*. The resultant plasmid was digested by *Spe*I and *Sal*I, and the *sod2*::*ADE2* cassette was used to disrupt the *SOD2* gene of *S. cerevisiae* YPH250. Disruption of the *SOD2* gene was verified by PCR and activity staining of Mn-SOD by the method of Beauchamp and Fridovich [23].

The open reading frame of *CCP1* (cytochrome *c* peroxidase) of *S. cerevisiae* S288C was amplified by PCR using the following primers: 5′-CCTGAGTACTGAGTAATATG-3′ and 5′-TAAA-CCTTGTTCCTCTAAAGTCTTGAAAAT-3′. The amplified DNA fragment (1269 bp) was treated with Klenow and cloned into the *Hin*cII site of pUC19 to yield the plasmid pUC19+*CCP1*. The plasmid (pUC19+*CCP1*) was treated with *Apa*I and *Nco*I followed by Klenow, and the *HIS3* gene was inserted to give the plasmid pUC19-*ccp1*Δ::*HIS3*. The resultant plasmid was digested by *Pvu*II and *Sac*I, and the *ccp1*Δ::*HIS3* cassette was used to disrupt the *CCP1* gene of *S. cerevisiae* YPH250, which was verified by PCR.

Heat-shock treatment

Cells of YNN27 (wild type), YH1 (*gsh1*) and YL1 (*gsh2*) were cultured in a 200 ml flask containing 50 ml of SD minimal medium (2% glucose/0.67% yeast nitrogen base without amino acids, pH 5.5) supplemented with 20 μg/ml L-Trp and 20 μg/ml uracil at 23 °C. When the D_{610} value reached 0.1, 5 ml of the culture was transferred immediately to a test tube that had been kept at 48 °C in a water-bath incubator. Periodically, a small portion of the culture was taken, diluted and plated on to YPD (2% glucose/1% yeast extract/2% peptone, pH 5.5) agar. Cells were cultured at 28 °C for 4 days and colonies were counted.

Adaptation test

Cells were cultured at 23 °C in SD minimal medium until the D_{610} value reached 0.1 as described above, and the test tube containing the cell suspension was immediately put into an incubator heated to 37 °C. The cells were cultured for another 1 h at 37 °C and then transferred to an incubator preheated at 48 °C. A portion of the culture was taken periodically and cell viability was determined as described above.

Western-blot analysis

Cells of YNN27 (wild type), YH1 (*gsh1*) and YL1 (*gsh2*) were cultured in a flask containing 50 ml of SD minimal medium supplemented with 20 μg/ml L-Trp and 20 μg/ml uracil at 23 °C. When the D_{610} reached approx. 1.0, cells were put into a water-bath incubator preheated at 37 °C and cultured for another 1 h. After cultivation, cells were collected by centrifugation, washed once with 0.85% NaCl solution, suspended in 0.2 ml of 10 mM Tris/HCl buffer (pH 7.0) and the cell suspension was transferred into an Eppendorf microcentrifuge tube. An approximately equal volume of glass beads was added and cells were disrupted using a vortex mixer at maximum speed for 3 min. Cell homogenates were centrifuged at 12000 ***g*** for 15 min at 4 °C and the resultant supernatants (5 μg of protein) were subjected to SDS/PAGE [24]. Proteins were electrically transferred on to PVDF membrane (Immobilon, Millipore). Hsp104p was detected by anti-Hsp104p antiserum raised in rabbit (Funakoshi, Tokyo, Japan), and immunoreactive protein was revealed with horseradish peroxidase-conjugated secondary antibody (Jackson Immunoresearch Laboratories) and diaminobenzidine. Protein concentration was determined by the method of Bradford [25].

Catalase assay

Catalase activity was measured according to the method of Roggenkamp et al. [26]: 1 unit was defined as the amount of enzyme decomposing 1 μmol of H_2O_2 per min at 25 °C.

Detection of respiration-deficient (RD) mutant

To determine the population of RD mutants generated by heat shock in a *gsh1* mutant (YH1) and its parental wild-type strain (YNN27), cells were cultured in a 200 ml flask containing 50 ml of SD minimal medium at 23 °C until the D_{610} reached 0.1. Then, 5 ml of the culture was transferred immediately to a test tube that had been heated to 37 °C in a water-bath incubator. After 1 h of incubation at 37 °C, the tube containing cell suspension was shifted to 48 °C. A portion of the culture was taken periodically, diluted appropriately and plated on to YPD agar. Cells were incubated at 28 °C for 4 days and colonies were replica-plated on to SG (2% glycerol/0.67% yeast nitrogen base without amino acids supplemented with 20 μg/ml L-Trp and 20 μg/ml uracil, pH 5.5) agar. The ratio (%) of RD mutant was calculated as follows: [1−(the number of colonies formed on SG plate)/(the number of colonies formed on YPD plate)]×100.

Respiration deficiency of yeast was also detected by the tetrazolium-overlay method as described by Ogur et al. [27]. Briefly, cells of the wild type (YPH250) and *sod2*Δ and *ccp1*Δ mutants were cultured in YPG (2% glycerol/1% yeast extract/2% peptone, pH 5.5) medium to avoid accumulation of RD mutant in glucose medium. When the D_{610} reached 0.1, cells were collected by centrifugation, washed once with sterilized water and suspended in YPD medium containing various concentrations of antimycin A, with an D_{610} value of 0.1, and incubation was continued at 28 °C for another 1 h. A portion of the culture

was withdrawn, diluted appropriately and plated on to YPD agar. Cells were cultivated for 4 days at 28 °C to make colonies. After cultivation, 1 % agar containing 0.1 % 2,3,5-triphenyltetrazolium chloride was poured on to the colonies and the plates were kept at room temperature for 16 h. Colonies that did not turn red in colour, indicating respiration deficiency, were counted and the population of RD mutants (the white colonies) was expressed as a percentage of the total number of colonies appearing on the plate.

Treatment with buthionine sulphoximine (BSO)

Cells of the *sod2*Δ mutant and its isogenic wild-type strain (YPH250) were cultured in SD minimal medium containing various concentrations of BSO at 28 °C with reciprocal shaking. Cell growth was monitored by measuring D_{610}.

Treatment with 1-chloro-2,4-dinitrobenzene (CDNB)

Cells of *sod2*Δ mutant and the wild-type strain (YPH250) were cultured in YPG medium until D_{610} reached approx. 0.1–0.2. The cells were collected by centrifugation, washed once with sterilized water and suspended in YPD medium containing various concentrations of CDNB and incubated at 28 °C. A portion of the culture was withdrawn periodically and plated on to YPD agar. Cells were incubated for 4 days prior to counting of cell numbers.

RESULTS

GSH protects *S. cerevisiae* from cell death induced by heat shock

Previously we demonstrated that heat-shock stress enhanced oxygen respiration in *S. cerevisiae*, which resulted in increased cellular oxidation levels [2]. We also reported that the GSH content of the cells treated by heat shock was increased [2]. To get further insight into the physiological significance of the induction of GSH synthesis during the heat-shock stress response, we exposed yeast strains deficient in GSH biosynthesis to heat-shock stress and examined thermotolerance. *S. cerevisiae* strains YNN27 (*GSH1*$^+$ *GSH2*$^+$), YH1 (*gsh1*$^-$ *GSH2*$^+$) and YL1 (*GSH1*$^+$ *gsh2*$^-$) were exposed directly to heat-shock stress from 23 to 48 °C to examine the effect of the GSH deficiency on survival at lethal temperatures. As shown in Figure 1, cells died rapidly when they were transferred to 48 °C. The strains YH1 and YL1 died slightly faster than their parental wild-type strain YNN27; however, the survival rate was increased if GSH (1 mM) was added to the culture just before the heat-shock treatment (Figure 1). Strains YH1 (*gsh1*$^-$) and YL1 (*gsh2*$^-$) cannot produce GSH at all [22]. Therefore, these results suggest that GSH protects the yeast from cell death.

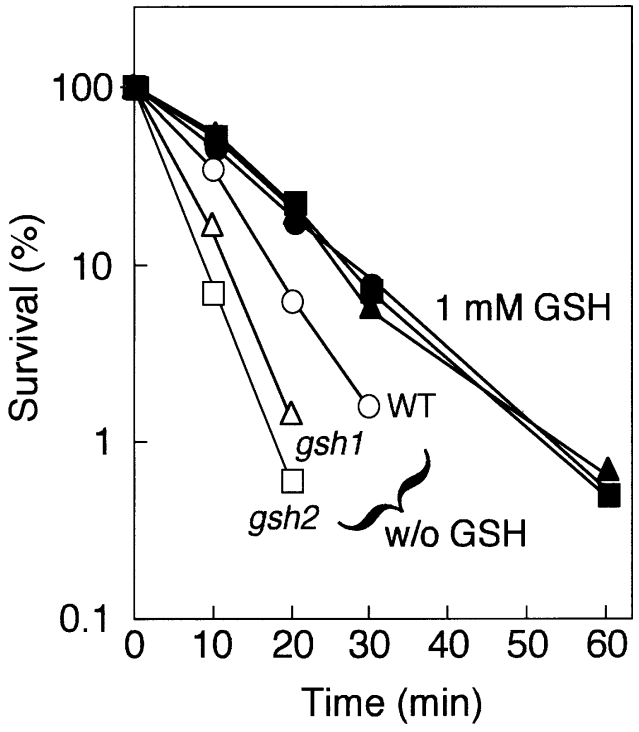

Figure 1 Effect of GSH on survival at lethal temperature

Cells were cultured at 23 °C in SD minimal medium until D_{610} reached 0.1, and the cells were shifted to 48 °C. A small portion of the culture was taken, diluted appropriately and plated on to YPD agar to determine cell viability. Open symbols, heat-shock treatment without (w/o) GSH; closed symbols, heat-shock treatment with 1 mM GSH. ●, ○, Wild-type (YNN27); ▲, △, *gsh1* mutant (YH1); ■, □, *gsh2* mutant (YL1). Results represent the means from three independent experiments. S.D. did not exceed 5% in the range 100–10%, 1% in the range 10–1% or 0.5% in the range of 1–0.1 %.

GSH is required for full acquisition of thermotolerance

If a cell that has been treated at sublethal temperature is shifted to lethal temperature, it acquires thermotolerance during the period of mild heat-shock stress and the viability of the cell after the shift to lethal temperature is higher than that of a cell that is exposed directly to severe stress. This phenomenon is called an adaptation to heat stress. Wild-type cells of *S. cerevisiae* (YNN27) acquired thermotolerance when they were pretreated at 37 °C for 60 min (Figure 2). Both of the GSH-deficient mutants (YH1 and YL1) could also acquire thermotolerance and showed higher survival rates, comparable with that of their parental wild-type strain, during the first 30 min for the *gsh1* mutant and the first 60 min for the *gsh2* mutant (Figure 2).

Hsp104p is a major HSP that confers resistance to heat-shock stress, because deletion of the *HSP104* gene increases sensitivity to higher temperature [28,29]. To investigate whether the GSH deficiency affects expression of *HSP* genes, levels of Hsp104p were determined as a representative of HSPs in this microorganism. As shown in Figure 3, no distinct difference was observed in the levels of Hsp104p in the wild type or *gsh1* and *gsh2* mutants after mild heat-shock stress. In addition to *HSP104*, deletion of *CTT1* has been known to cause increased sensitivity to heat shock [6]. We then measured catalase activity in the wild type and the GSH-deficient mutant cells. As shown in Figure 3, catalase was normally induced in all of these strains. These results indicate that the GSH deficiency may not affect induction of HSP and other heat-shock-inducible proteins.

Although the *gsh1* mutant showed the same viability as the wild-type strain during the first 30 min period at 48 °C after pretreatment at 37 °C, the cells died rapidly thereafter (Figure 2). Similarly, the *gsh2* mutant exhibited the same viability compared with that of wild-type cells during the first 60 min period and then died rapidly, whereas the wild-type cells still showed high viability. To assess whether GSH was required for *gsh1* and *gsh2* mutants to survive after the first 30 and 60 min periods, respectively, at lethal temperature after pretreatment with a sublethal temperature, we added GSH (1 mM) to the culture at the end of the adaptation period, or after exposure to the lethal

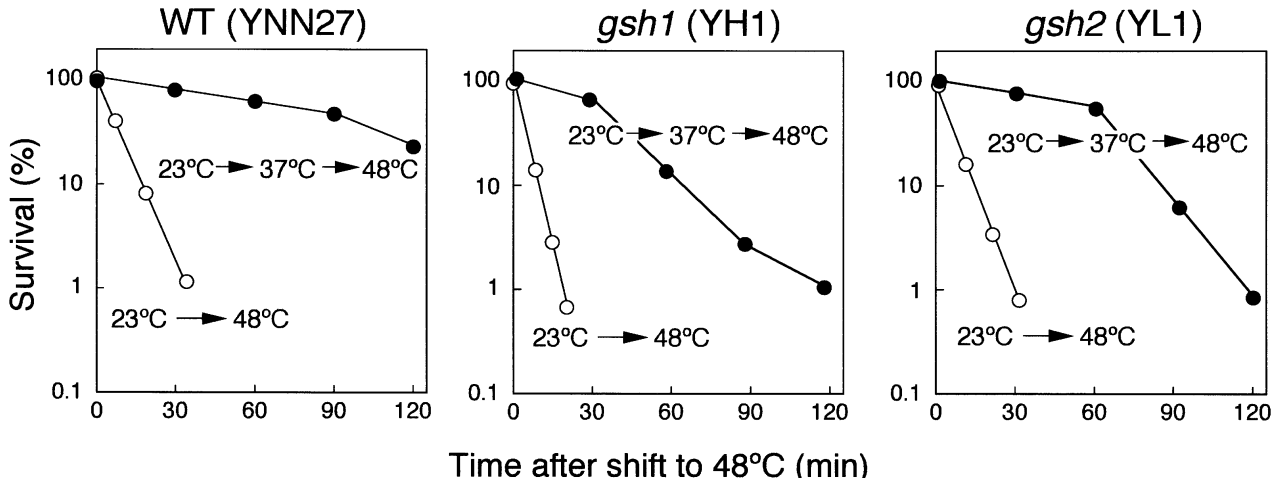

Figure 2 GSH deficiency does not affect acquisition of thermotolerance

Cells were cultured at 23 °C in SD minimal medium until D_{610} reached 0.1 and shifted to 37 °C. Cells were cultured for another 1 h at 37 °C and then shifted to 48 °C. Cell viability after the shift to 48 °C was monitored by counting the colonies on YPD agar plates. ○, Cells were directly shifted from 23 to 48 °C; ●, cells were shifted to 48 °C after pretreatment at 37 °C for 1 h. Viability of cells before shift to 48 °C was taken as 100%. Results represent the means from three independent experiments. S.D. did not exceed 5% in the range 100–10%, 1% in the range 10–1% or 0.5% in the range 1–0.1%.

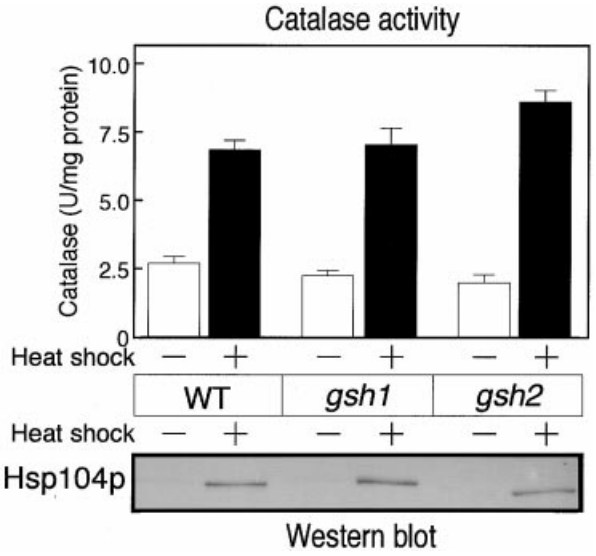

Figure 3 Induction of catalase and Hsp104p by heat-shock stress in GSH-deficient mutants

Cells (WT, YNN27; *gsh1*, YH1; *gsh2*, YL1) were cultured at 23 °C in SD minimal medium until D_{610} reached 1.0, shifted to 37 °C, and culture was continued for another 1 h. Experimental procedures are described in the Materials and methods section. Data on catalase activity are from three independent experiments (means ± S.D.).

temperature. As shown in Figure 4, the addition of GSH to *gsh1* mutant just before the shift to 48 °C gave no effect in terms of thermotolerance during the first 30 min period, but it improved viability during the period between 30 and 60 min. However, after a 60 min period both the GSH-treated cells and the GSH-untreated cells died in the same rates. On the other hand, cell viability was not improved if GSH was added 30 min later when the temperature was shifted from 37 to 48 °C. The *gsh2* mutant showed a similar tendency in the acquisition of thermotolerance by the addition of GSH. If GSH was added 30 min after the shift to 48 °C, viability after the first 60 min period was slightly improved. The viability at lethal temperature was increased to a much greater extent if GSH was added at the end of the 37 °C adaptation period (Figure 4). However, if GSH was added 60 min after the shift to 48 °C, no effect was observed (results not shown). These results lead us to propose that GSH may play some important roles in the early events that may happen during the first 30 min period at lethal temperature but that it can no longer prevent cell death once the catastrophe has begun.

Glutathione deficiency causes generation of RD mutants by heat shock

Mitochondria are organelles in which large amounts of reactive oxygen species are expected to be generated by respiration; therefore, it is highly probable that mtDNA receives oxidative damage. In *S. cerevisiae*, a defect in the mtDNA causes RD mutants. To explore the early events that may happen in a *gsh1* mutant at lethal temperature after pretreatment with sub-lethal temperature, we measured populations of RD mutants. As shown in Figure 5, numbers of RD mutants increased in the *gsh1* mutant after heat-shock treatment, in contrast to the wild type. To assess whether the increase of RD mutants in the *gsh1* mutant strain was due to a lack of GSH in the cells, GSH was added just before the shift from 37 to 48 °C. Generation of RD mutants was found to be partially suppressed by the addition of GSH (Figure 5). In the case of the *gsh2* mutant, the ratio of RD mutants did not increase. This is presumably due to accumulation of γ-L-glutamyl-L-cysteine (γ-GC), an intermediate of GSH synthesis, in the *gsh2* mutant [22,30]. This will be discussed below. These results suggest that GSH protects mtDNA from the oxidative damage caused by heat-shock stress.

Depletion of GSH in a *sod2*Δ mutant induces cell death

Taking these observations together with our previous findings, *S. cerevisiae* may synthesize GSH as one of the adaptive responses

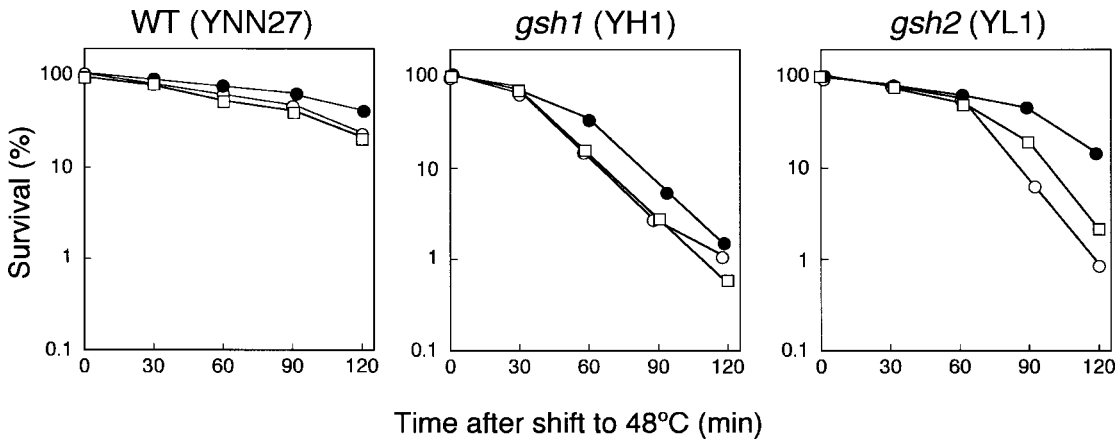

Figure 4 Effect of GSH on thermotolerance

Cells were cultured at 23 °C in SD minimal medium. When the D_{610} reached 0.1, cells were shifted to 37 °C and incubation was continued for another 1 h. ○, Cells were shifted to 48 °C after treatment at 37 °C for 1 h; ●, 1 mM GSH was added after 1 h of incubation at 37 °C and the cells were shifted to 48 °C; □, 1 mM GSH was added 30 min after the shift to 48 °C. Results represent the means from three independent experiments. S.D. did not exceed 5% in the range 100–10%, 1% in the range 10–1% of 0.5% in the range 1–0.1%.

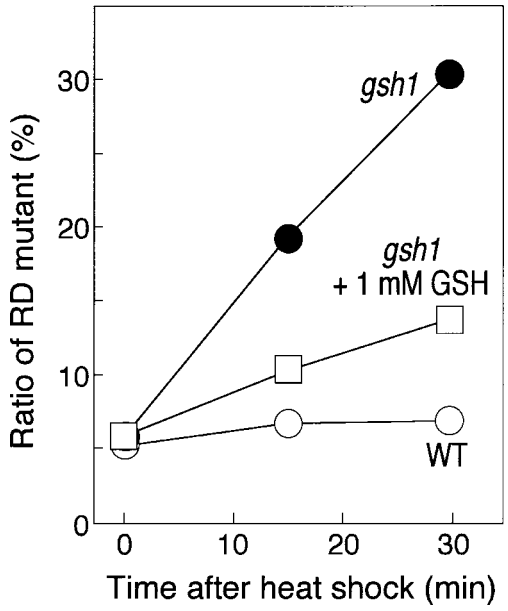

Figure 5 Effect of heat shock on generation of RD mutants

Cells (WT, YNN27; *gsh1*, YH1) were cultured at 23 °C in SD minimal medium until D_{610} reached 0.1 and shifted to 37 °C. After 1 h of incubation at 37 °C, the cells were transferred to 48 °C. A portion of the culture was taken periodically, diluted and plated on to YPD agar. The ratio of RD mutants was calculated as described in the Materials and methods section. ○, wild type; ●, *gsh1* mutant; □, *gsh1* mutant with 1 mM GSH, which was added just before the shift to 48 °C.

to heat-shock stress to protect the mtDNA from oxidative damage that is caused by increased levels of oxygen respiration in this organelle. Exogenously added GSH is likely to protect the mtDNA from oxidative damage; however, once catastrophe has begun GSH may no longer prevent it. To assess this possibility, we disrupted the *SOD2* gene encoding mitochondrial Mn-SOD and then depleted intracellular GSH in the resulting *sod2*Δ mutant by BSO to mimic such a situation.

The steady-state rate for spontaneous generation of RD mutants in the *sod2*Δ mutant was approx. four times higher than in the wild-type strain (Figure 6A). Since antimycin A specifically blocks electron transport from cytochrome *b* to *c* in complex III in the mitochondrial electron-transport chain, ubiquinone transfers an electron to molecular oxygen to generate reactive oxygen species [31]. As shown in Figure 6(A), the population of RD mutants in the *sod2*Δ strain was increased in accordance with the concentration of antimycin A. This presumably reflects the accumulation of oxidative damage in the mtDNA due to the lack of Mn-SOD in the mitochondrial matrix where the mtDNA is present. On the other hand, disruption of *CCP1* (cytochrome *c* peroxidase) did not enhance the generation of RD mutants to the same extent as disruption of Mn-SOD. Cytochrome *c* peroxidase is localized to the intermembrane space of mitochondria [32–34]; thus this enzyme might not contribute so much to the protection of mtDNA from oxidative damage.

Next, the effect of antimycin A on survival of the *sod2*Δ mutant was examined. For the concentration (200 μM) we tested, no distinct difference was observed in the survival rates between the wild type and the *sod2*Δ mutant (Figure 6B). We then examined cell growth of the *sod2*Δ mutant in the medium containing BSO, which inhibits GSH synthesis. As shown in Figure 7, the *sod2*Δ mutant could not grow in the medium containing 1.0 mM BSO, whereas wild-type cells could grow. We also examined the effect of CDNB on cell viability of the *sod2*Δ mutant. Treatment of yeast cells with 1 mM CDNB for 60 min reduced intracellular GSH content (untreated, 2.22 ± 0.04 μmol/g of cells; treated, 1.23 ± 0.08 μmol/g of cells). Cell viability of the wild-type strain after treatment with CDNB was not changed, whereas it was reduced to approx. 1.2% of untreated cell viability in the case of the *sod2*Δ mutant (results not shown). These results suggest that the depletion of GSH in cells with dysfunctional mitochondria or in cells with insufficient ability to scavenge reactive oxygen species in mitochondria may induce cell death.

DISCUSSION

Although GSH-deficient mutants died rapidly if the cells were exposed directly to lethal temperature, exogenously added GSH

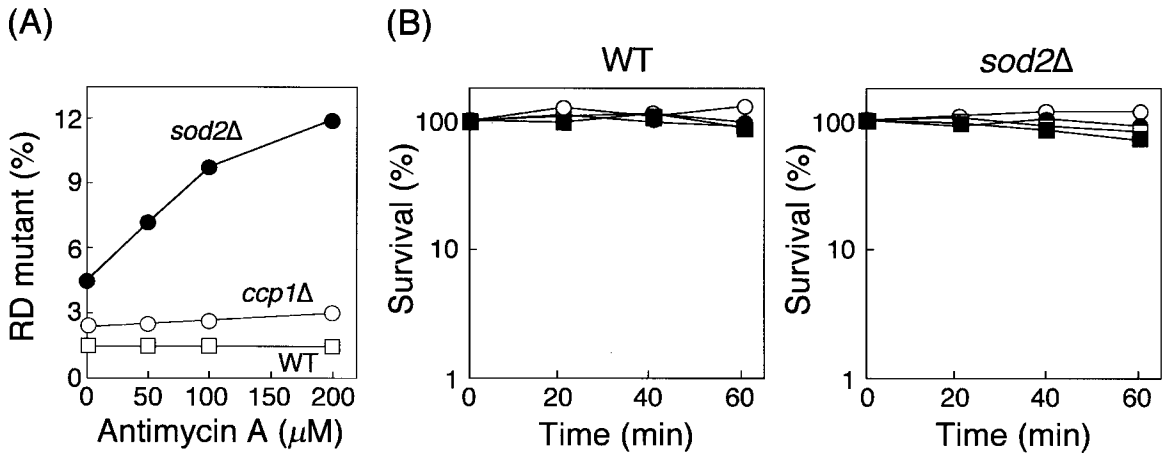

Figure 6 Effect of deletion of Mn-SOD on the generation of RD mutants

(**A**) Cells of the wild type (YPH250) and *sod2*Δ and *ccp1*Δ mutants were cultured in YPG medium until D_{610} reached 0.1. Cells were collected, washed once with sterilized water and suspended in YPD medium containing various concentrations of antimycin A. Incubation was continued at 28 °C for another 1 h. Generation of RD mutants was calculated as described in the Materials and methods section. (**B**) Cells were treated with various concentrations of antimycin A (○, 0 μM; ●, 50 μM; □, 100 μM; ■, 200 μM) as described above. A portion of the culture was taken, diluted appropriately and plated on to YPD agar to measure viability.

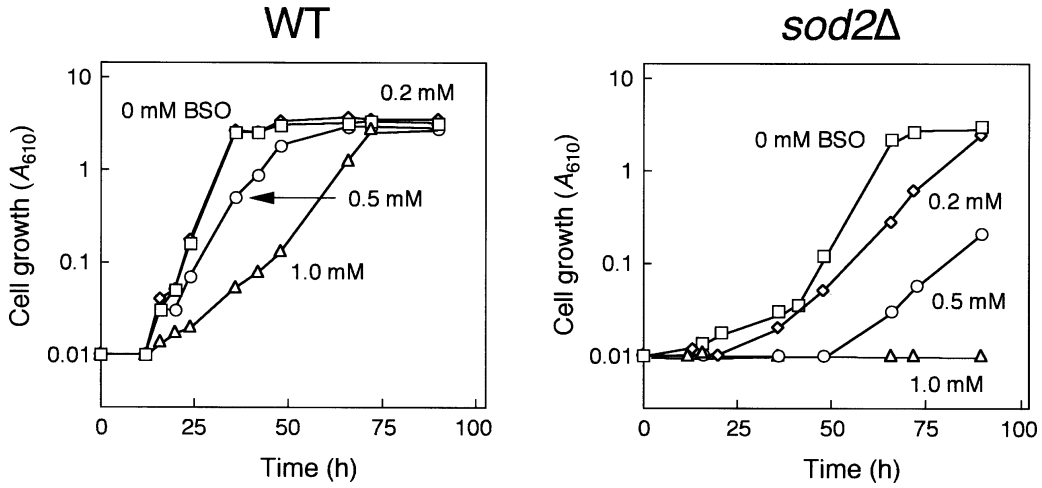

Figure 7 Effect of GSH depletion on cell growth of the *sod2*Δ mutant

Cells were cultured in SD minimal medium containing various concentrations of BSO as indicated in the Figure. Cell growth was monitored by measuring D_{610} periodically.

could suppress the cell death induced by heat shock (Figure 1). As shown in Figure 2, the *gsh2* mutant that had been pretreated at 37 °C for 60 min showed high viability during the first 60 min upon the shift to lethal temperature, whereas viability of the *gsh1* mutant after 30 min was decreased under the same conditions. Ohtake et al. [22] and Grant et al. [30] reported that the *gsh2* mutant accumulated high concentrations of γ-GC. γ-GC is an intermediate in GSH biosynthesis, i.e. Gsh1p catalyses synthesis of γ-GC from L-Glu and L-Cys, and Gsh2p synthesizes GSH from γ-GC and Gly. Grant et al. [30] reported that γ-GC could partially substitute for GSH in oxidative-stress resistance. We also demonstrated that γ-GC could substitute for GSH in the GSH-dependent detoxification of methylglyoxal both *in vivo* and *in vitro* [35]. Furthermore, since we found that exogenously added γ-GC could partially suppress heat-shock-induced cell death (results not shown), γ-GC might function as an alternative for GSH. Anyway, GSH seems important in delaying cell death at lethal temperature, although it may not necessarily be required for the acquisition of thermotolerance in adaptation to heat shock.

Our data led us to propose that the heat-shock response of *S. cerevisiae* can be divided into two steps. The first step is the induction period of HSPs and other heat-inducible proteins. The second step is the period for such HSPs to work and for metabolic change, including that involving GSH and other antioxidant enzymes. GSH may not necessarily be required for induction of HSPs, although in the second step GSH may play an important role in maintaining cell viability at higher temperatures. For example, glutathione S-transferase, which uses GSH, may be involved in this step [20]. In addition, other antioxidant enzymes

such as cytosolic catalase and Tsa1p may also be involved in this step, because the mutants deficient in these enzymes showed increased susceptibility to heat shock [4,6]. In contrast, Saunders et al. reported that Chinese hamster ovary cells depleted of GSH did not induce HSPs [36]. In addition, Rokutan et al. [37] reported that GSH depletion inhibited the nuclear translocation of HSF1 (heat-shock transcription factor 1) after exposure to heat stress in guinea pig gastric mucosal cells. However, we have demonstrated that induction of Hsp104p was normal in the GSH-deficient *S. cerevisiae* cells (Figure 3). In higher eukaryotes like human and *Drosophila melanogaster*, heat-shock transcription factor is translocated to the nucleus by heat shock, and then it binds to the heat-shock element in the HSP promoter [38]. In *S. cerevisiae*, Hsf1p binds constitutively to the heat-shock element [39]. Differences in the behaviour of heat-shock transcription factor may partially account for the viability of the GSH-deficient mutants of *S. cerevisiae* during the first 30–60 min after the shift from sublethal to lethal temperature.

Insufficient ability to scavenge reactive oxygen species in mitochondria may cause accumulation of oxidative damage in the mtDNA that results in the generation of RD mutants. Piper [40] reported that a null mutation of *SOD2* caused a higher frequency of mitochondrial mutation. We found that the steady-state rate for spontaneous generation of RD mutants of the *sod2δ* strain was approx. four times higher than for the wild-type cells (Figure 6A). Furthermore, we have demonstrated that cell growth was inhibited if GSH was depleted in the *sod2δ* background (Figure 7); however, antimycin A treatment (200 μM for 60 min) did not induce cell death (Figure 6B). These results may suggest that mitochondrial dysfunction is not enough to induce cell death, although GSH depletion triggers the catastrophic process. Madeo et al. [41] reported that depletion of GSH induced apoptosis in *S. cerevisiae*. They showed that accumulation of reactive oxygen species caused by depletion of GSH in the cell triggered the apoptotic pathway in this micro-organism and the hypoxia or scavenging of the radicals prevented apoptosis. Their observations support our hypothesis that GSH protects the yeast from heat-shock-induced cell death.

Besides GSH, thioredoxin (TRX) is also a major redox buffer in the cell. *S. cerevisiae* has three TRX genes (*TRX1*, *TRX2* and *TRX3*) [42,43]. TRX has clustered cysteine residues (CXXC), termed the TRX motif, in its active site. TRX belongs to the thiol-disulphide oxidoreductase family, and thus it can reduce many oxidized proteins. TRX can serve as an electron donor for TPx. TPx reduces H_2O_2 and organic peroxides such as t-butyl hydroperoxide. Park et al. [44] reported that *S. cerevisiae* has five functional TPxs; i.e. three in cytosol (cTPxI/Tsa1p, cTPxII/YDR453c, cTPxIII/Ahp1p), one in mitochondria (mTPx/YBL064c) and one in the nucleus (nTPx/Dot5p), while Lee et al. [45] suggested that Ahp1p was localized to peroxisomes. Recently Pedrajas et al. [46] reported that mTPx (which they named Prx1p) is a TPx in mitochondria where Trx3p, which is also localized to mitochondria [43], functions as an electron donor [46]. They also reported that disruption of the *PRX1* gene enhanced susceptibility to heat shock as well as oxidative stress [46].

Taken together, an increase in the generation of reactive oxygen species in mitochondria due to the lack of antioxidant system (Mn-SOD and TPx/Prx1p–TRX/Trx3p system) or a higher respiration rate caused by heat shock may accumulate oxidative damage in the mtDNA, which causes mitochondrial dysfunction and in turn induces further generation of oxygen radicals. Collapse of intracellular redox balance eventually induces cell death and GSH may partially prevent or delay the early events of this process.

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