Adaptive response of *Schizosaccharomyces pombe* to hydrogen peroxide and menadione

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The response of *Schizosaccharomyces pombe* to oxidative stresses has been examined. On challenging *Schiz. pombe* for 60 min at early exponential phase with either 40 mM H₂O₂ or 6 mM menadione (MD), a superoxide-generating agent, less than 10% of the cells survived. Pretreating *Schiz. pombe* cells with 0.2 mM H₂O₂ or 0.2 mM MD for 1 h significantly increased survival of these lethal doses of each oxidant, indicating the existence of an adaptive response to oxidative stress. Furthermore, cells pretreated with a low dose of MD became resistant to a lethal dose of H₂O₂. However, cells pretreated with H₂O₂ became only partially resistant to a lethal dose of MD. Adaptation was accompanied by the induction of several oxidative defence enzymes. The presence of 0.2 mM H₂O₂ induced catalase by 2.8-fold and peroxidase by 2.0-fold. The presence of 0.2 mM MD induced catalase by 2.0-fold, glucose-6-phosphate dehydrogenase by 1.9-fold, glutathione reductase by 2.7-fold, peroxidase by 3.0-fold, and superoxide dismutase (SOD) by 2.1-fold. The higher induction of these defence enzymes by MD may explain why MD-pretreated cells were better adapted to lethal doses of oxidants than H₂O₂-pretreated ones. All these enzymes except SOD and peroxidase increased more than 5.0-fold as cells proceeded into stationary phase. The GSH/GSSG ratio also increased by 60%. These changes accord with the observation that stationary phase cells survive oxidant treatment better than cells in vegetative growth.

Keywords: *Schizosaccharomyces pombe*, oxidative stress, hydrogen peroxide, menadione, adaptive response

INTRODUCTION

In order to cope with reactive oxygen species, organisms have evolved appropriate defences. Much progress has been made toward understanding the genetics and physiological responses to oxidative stress, especially in bacteria. Adaptive responses against H₂O₂ have been demonstrated in many bacteria (Christman *et al.*, 1985; Bol & Yasbin, 1990; Lee *et al.*, 1993), in *Saccharomyces cerevisiae* (Collinson & Dawes, 1992; Jamieson, 1992) and in mammalian cells (Spitz *et al.*, 1987). The global response to oxidative stress is best understood in *Escherichia coli* and *Salmonella typhimurium* (Farr & Kogoma, 1991; Storz *et al.*, 1990b) in which special regulators and/or redox sensors such as OxyR and SoxR respond to H₂O₂ or the superoxide

radical, respectively (Storz *et al.*, 1990a; Hidalgo & Dimple, 1994). Other bacteria also have redox-controlled regulator-sensor systems (Allen, 1993). Several anti-oxidant enzymes are known to be under the control of these regulators. Hydroperoxidase I (HPI, encoded by *katG*) and a subunit of alkylhydroperoxide reductase (encoded by *ahpF*) of *E. coli* are induced in an OxyR-dependent manner. MnSOD (encoded by *sodA*) and glucose-6-phosphate dehydrogenase (encoded by *zwf*) are induced by the superoxide radical through SoxRS (Farr & Kogoma, 1991; Storz *et al.*, 1990b). Antioxidant enzymes are also regulated by other stresses including starvation (Mukhopadhyay & Schellhorn, 1994) and heat shock (Christman *et al.*, 1985).

As a model eukaryote, *Sacch. cerevisiae* has been studied and shown to possess adaptation mechanisms against oxidative treatment (Collinson & Dawes, 1992; Jamieson, 1992; Flattery-O'Brien *et al.*, 1993) and to regulate antioxidant enzymes (Schnell *et al.*, 1992; Gralla &

Abbreviations: MD, menadione (2-methyl-1,4-naphthoquinone); G6PD, glucose-6-phosphate dehydrogenase; GR, glutathione reductase; SOD, superoxide dismutase

Kosman, 1992; Galiazzo & Labbe-Bois, 1993). Two catalases of *Sacch. cerevisiae*, a cytosolic catalase T (encoded by *CTT1*) and a peroxisomal catalase A (encoded by *CTA1*), are regulated by both O_2 (Hörtner *et al.*, 1982) via the transcription factor Hap1 (Zitomer & Lowry, 1992) and nutrients (Ruis & Hamilton, 1992). Both superoxide dismutases (SODs) in *Sacch. cerevisiae* (cytoplasmic Cu/ZnSOD and mitochondrial MnSOD) are induced by paraquat, a redox cycling agent (Galiazzo & Labbe-Bois, 1993). H_2O_2 can also induce Cu/ZnSOD in this organism (Schnell *et al.*, 1992). Yap1, a yeast homologue of mammalian AP-1, is known to activate the *TRX2* gene encoding thioredoxin (Kuge & Jones, 1994), its action increasing resistance to peroxide stress (Schnell *et al.*, 1992).

Since the fission yeast *Schizosaccharomyces pombe* obtains energy via respiration or fermentation it, like *Sacch. cerevisiae*, may encounter either hypo- or hyperoxic conditions (McDonald & Tsai, 1989). Here, we report the characteristics of adaptation against oxidative treatment with either H_2O_2 or menadione, a superoxide-generating agent in fission yeast, and the levels of induction of antioxidant enzymes in response to these oxidants. We also show that various growth conditions change the levels of these enzymes and the GSH/GSSG ratio, which in turn may affect the resistance of cells to oxidative stresses.

METHODS

Yeast strains, media and chemicals. *Schiz. pombe* strain ED665 (*h⁻, ade6-M210 leu1-32 ura4-D18*) was kindly provided by Dr Kyung-Hun Kim (Kangwon National University, Korea). *Sacch. cerevisiae* strain BWG1-7A (*MATa leu2-3 leu2-112 his4-519 ade1-100 ura3-52*) was used. YEPD medium containing 1% (w/v) yeast extract (Difco), 2% (w/v) bacto-peptone (Difco), and 2% (w/v) dextrose (Junsei) was used for cell growth. To produce fresh inocula, cells were grown in YES medium (Moreno *et al.*, 1991; 0.5% yeast extract, 3% dextrose, 250 mg supplements l^{-1}) overnight. All the chemicals used for assay of antioxidant enzymes were obtained from Sigma. Glutathione reductase (EC 1.6.4.2) for determination of cellular glutathione concentrations was also from Sigma and 2-vinylpyridine was purchased from Aldrich.

Determination of survival rates. Cells were grown to an OD_{595} of 0.15, which corresponds to about 2.4×10^6 viable cells ml^{-1} , and harvested by centrifugation at 5000 *g* for 5 min. They were resuspended in 100 mM potassium phosphate buffer (pH 7.0) to the same cell density and treated with oxidants at various concentrations. At 20 min intervals, aliquots were taken, diluted in 50 mM potassium phosphate buffer and plated on YES agar plates in duplicate. To monitor adaptation, cells were pretreated with an appropriate concentration of oxidants in YEPD medium for 60 min at 30 °C with shaking. They were harvested, resuspended in 100 mM potassium phosphate buffer and challenged with higher concentrations of oxidants. Aliquots were taken at 15 min intervals, diluted and plated on YES agar in duplicate. To monitor the survival of cells from different growth phases, samples of a culture were taken at an OD_{595} of 0.15 for early exponential phase, an OD_{595} of 2.5 for late exponential phase and 72 h after inoculation (OD_{595} of about 5.0) for stationary phase. Cells were harvested and washed as above. Cells from later growth phases were diluted in potassium

phosphate buffer to an OD_{595} of about 0.15. Subsequent challenge with oxidant and plating were as above.

Preparation of cell-free extracts. Harvested cells were washed in 10 mM potassium phosphate buffer, resuspended in 50 μl 10 mM potassium phosphate buffer containing 1 mM PMSF and disrupted by abrasion with glass beads in Eppendorf tubes using a vortex mixer. Cell debris was removed by two consecutive centrifugations and the amount of protein in the supernatant was determined by the Bradford assay (Bradford, 1976).

Enzyme assays. Catalase activity was determined as described by Beers & Sizer (1952). The rate of disappearance of H_2O_2 was measured spectrophotometrically at 240 nm. One unit of enzyme was defined as the activity that catalyzed the degradation of 1 μmol H_2O_2 min^{-1} . The activity of glucose-6-phosphate dehydrogenase (G6PD) was determined according to the protocols outlined by Decker (1977). The rate of reduction of $NADP^+$ was monitored by absorption at 340 nm. One unit of enzyme activity was defined as the activity that catalyzed the reduction of 1 μmol $NADP^+$ min^{-1} . Glutathione reductase (GR) activity was measured by the procedure of Smith *et al.* (1988). The assay was based on the reduction of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) by reduced glutathione (GSH), which is produced by GR. Absorption at 412 nm was monitored for more than 5 min to ensure that the crude extract was depleted of any substrates capable of reducing DTNB. Then 1 mM oxidized glutathione (GSSG) was added and the increase in absorption at 412 nm was measured. One unit of enzyme was defined as the amount of activity that catalysed the production of 1 μmol thiobis(2-nitrobenzoic acid) (TNB) min^{-1} . General peroxidase activity was measured spectrometrically by monitoring the oxidation of *o*-dianisidine at 460 nm in the presence of H_2O_2 according to the Decker (1977). One unit of enzyme activity was defined as the activity that increased the OD_{460} by 0.01 min^{-1} . Total SOD activity was determined by the chemical method based on NADH oxidation described by Paoletti & Mocali (1993). The reaction mixture contained 40 μl 7.5 mM NADH, 25 μl 100 mM EDTA/50 mM $MnCl_2$, an appropriate amount of extract, and 0.1 ml 10 mM β -mercaptoethanol in 0.9 ml triethanolamine/diethanolamine/HCl (100 mM each) buffer. The change at OD_{340} was recorded for 15 min. The blank was measured in a cuvette where the sample was replaced with an equal volume of buffer. One unit of SOD activity was defined as the amount of enzyme required to inhibit the rate of NADH oxidation of the control by 50%. MnSOD (cyanide-insensitive) was distinguished from Cu/ZnSOD activity by inclusion of 2 mM CN^- in the assay. SOD activity was calculated using the calibration table presented by Paoletti & Mocali (1993).

Determination of cellular GSH and GSSG. Cellular levels of GSH and GSSG were determined using GR and 2-vinylpyridine as described by Griffith (1980). Total glutathione in cell extracts was determined by reducing GSSG to GSH with GR and measuring the rate of reduction of DTNB to TNB by GSH. The amount of GSSG was determined after masking GSH with 2-vinylpyridine. A standard curve was obtained by correlating known amounts of glutathione with the rates of DTNB reduction.

RESULTS AND DISCUSSION

Resistance of *Schiz. pombe* to oxidative stresses

Exponentially growing *Schiz. pombe* cells were treated with various concentrations of H_2O_2 . Cell survival was not affected significantly by concentrations up to 10 mM

Fig. 1. Survival rates of *Schiz. pombe* after treatment with H_2O_2 or MD. Exponentially growing cells at an OD_{595} of 0.15 were treated with H_2O_2 (a) or MD (b). At 20 min intervals, aliquots were taken and plated on YES in duplicate to monitor cell viability. Concentrations of H_2O_2 were 0 ($\circ$), 0.2 ($\bullet$), 10 (∇), 40 ($\blacktriangledown$) or 100 mM ($\square$). Concentrations of MD were 0 ($\circ$), 0.2 ($\bullet$), 4 (∇), 6 ($\blacktriangledown$), 15 ($\square$) or 30 mM ($\blacksquare$). Data are from one representative experiment.

(Fig. 1a). At 40 mM H_2O_2 , less than 10% of cells survived after 1 h of treatment. The requirement for such a high concentration of H_2O_2 for killing is unusual compared with other systems. *E. coli* is killed at a H_2O_2 concentration of 1–3 mM, mainly because of DNA damage (Imlay & Linn, 1988). *Streptomyces coelicolor*, a strict aerobe, exhibits 1% survival when treated with 20 mM H_2O_2 for 20 min (Lee *et al.*, 1993). *Sacch. cerevisiae* shows sensitivity to only about 2 mM H_2O_2 (Collinson & Dawes, 1992; Jamieson, 1992). The resistance of *Schiz. pombe* to H_2O_2 may be partly ascribed to a lack of lipid peroxidation (Janda *et al.*, 1993) and higher levels of several antioxidant enzymes, as well as GSH, as demonstrated below. In contrast, menadione (MD) markedly affected *Schiz. pombe* cell survival at 6 mM (Fig. 1b), the range of MD concentrations achieving appreciable killing being comparable to that observed for *Sacch. cerevisiae* (Flattery-O'Brien *et al.*, 1993).

Adaptation to oxidative stresses

An organism treated with a nonlethal dose of a stress condition can usually withstand subsequent higher doses of the same stress. Similar adaptation can also result from treatment with different kinds of stresses (Farr & Kogoma, 1991). To examine whether *Schiz. pombe* can adapt to oxidative stresses, we tested the effect of sublethal doses of H_2O_2 or MD on the resistance of cells to subsequent exposure to lethal doses of these oxidants. Exponentially growing cells were treated with a sublethal concentration (0.2 mM) of each oxidant for 1 hour in YEPD medium and subsequently treated with a lethal concentration of each oxidant (40 mM H_2O_2 or 6 mM MD) in phosphate buffer. Cells pretreated with H_2O_2 or MD could tolerate the lethal dose of H_2O_2 (Fig. 2a). The extent of resistance was high, which indicates that in the

Fig. 2. Adaptation of *Schiz. pombe* to oxidative stresses. Exponentially growing cells in YEPD medium were not pretreated (∇), or were pretreated with 0.2 mM H_2O_2 ($\bullet$) or 0.2 mM MD ($\blacktriangledown$) for 60 min. They were then challenged with either 40 mM H_2O_2 (a) or 6 mM MD (b). At 15 min intervals, aliquots were taken and plated on YES in duplicate. The survival of untreated cells was monitored as a control ($\circ$). Data are from one representative experiment.

pretreated cells almost full protection against the applied H_2O_2 stress was induced. This demonstrates the existence of an adaptive response by which *Schiz. pombe* acquires resistance against H_2O_2 stress following pretreatment with either H_2O_2 or MD.

In the case of killing with MD, however, the extent of the cellular response to pretreatment with H_2O_2 and MD was different (Fig. 2b). Cells pretreated with MD showed relatively high resistance to subsequent challenges with a lethal dose of MD, their survival increasing about 8-fold. On the other hand, cells pretreated with H_2O_2 and then exposed to lethal MD stress showed only a slightly increased resistance (Fig. 2b). This demonstrates that although cells pretreated with each oxidant became more resistant to MD stress, pretreatment with H_2O_2 was not very effective in causing adaptation to MD stress. Jamieson (1992) observed a similar tendency in *Sacch. cerevisiae*, although there is also a contradictory report that *Sacch. cerevisiae* became more resistant to MD stress after pretreatment with H_2O_2 , but not vice versa (Flattery-O'Brien *et al.*, 1993). If superoxide radicals produced by MD are dismutated into hydrogen peroxide inside the cell, then it is reasonable to propose that superoxide generators can induce a system of protection triggered by H_2O_2 . This may be why *Schiz. pombe* cells treated with a low dose of MD are fully protected against H_2O_2 stress whereas H_2O_2 -pretreated cells cannot evoke full protection against MD stress.

The above observations suggest that the superoxide radical, although it probably has some cytotoxic effect, can be a better signalling molecule to prepare cells against oxidative stress in *Schiz. pombe*. Further investigation of *in vivo* concentrations of various reactive oxygen species in *Schiz. pombe* following treatment with H_2O_2 or MD is necessary.

Table 1. Changes in specific activities of oxidative defence enzymes in *Schiz. pombe* and *Sacch. cerevisiae* depending on oxidant treatment and growth phase

Cells were grown as described in Methods. Exponentially growing cultures were treated with 0.2 mM H₂O₂ or 0.2 mM MD for 1 h and cell-free extracts prepared. The values presented are means $\pm$ SD from 4 independent cultures and are expressed in U (mg protein)⁻¹, with the exception of G6PD, which is expressed in mU (mg protein)⁻¹.

Enzyme	<i>Schiz. pombe</i>						<i>Sacch. cerevisiae</i>
	Early exponential			Late exponential	Stationary	Early exponential	Early exponential
	Oxidant...	–	H ₂ O ₂	MD	–	–	
Catalase		30.0 $\pm$ 4.6	84.9 $\pm$ 5.7	60.8 $\pm$ 1.9	98.6 $\pm$ 11.4	141.0 $\pm$ 21.0	4.0 $\pm$ 0.5
G6PD		33.5 $\pm$ 4.3	34.3 $\pm$ 7.2	62.1 $\pm$ 2.4	97.7 $\pm$ 10.2	191.5 $\pm$ 4.0	202.6 $\pm$ 12.5
GR		22.3 $\pm$ 1.1	27.6 $\pm$ 1.8	60.7 $\pm$ 7.8	44.7 $\pm$ 5.6	122.4 $\pm$ 20.3	39.7 $\pm$ 5.6
Peroxidase		3.1 $\pm$ 0.2	6.2 $\pm$ 0.5	9.2 $\pm$ 0.7	4.3 $\pm$ 0.2	7.4 $\pm$ 0.4	3.5 $\pm$ 0.2
SOD		40.3 $\pm$ 5.2	45.3 $\pm$ 6.2	83.4 $\pm$ 7.5	40.0 $\pm$ 7.3	39.5 $\pm$ 5.2	13.7 $\pm$ 1.3

Antioxidant enzymes are induced at different levels by H₂O₂ and MD

To investigate the mechanism of adaptation, we measured the changes in the levels of several antioxidant enzymes in early exponential phase cells pretreated with sublethal dose of each oxidant (Table 1). Treatment with 0.2 mM H₂O₂ induced catalase and peroxidase 2.8- and 2.0-fold, respectively. The levels of other enzymes increased only marginally. However, the presence of 0.2 mM MD increased the level of these enzymes to a greater extent. Catalase, G6PD and SOD increased approximately 2-fold, whereas GR and peroxidase increased 3-fold. The levels of cyanide-sensitive (possibly Cu/ZnSOD) and cyanide-insensitive (possibly MnSOD) SOD activity increased equally after treatment with MD (data not shown). The greater induction of antioxidant enzymes by 0.2 mM MD compared to 0.2 mM H₂O₂ may be responsible for the better protection against oxidative stress induced by MD (Fig. 2). The induction of mainly catalase and peroxidase by 0.2 mM H₂O₂ might be sufficient to protect against a lethal dose of H₂O₂, whereas induction of additional enzymes such as G6PD, GR and SOD may be necessary to protect cells against superoxide stress.

The level of the same antioxidant enzymes in *Sacch. cerevisiae* at early exponential phase was measured (Table 1). *Sacch. cerevisiae* contained a higher level of G6PD but much lower levels of catalase and SOD. The difference in the level of catalase may explain why *Schiz. pombe* is more resistant to H₂O₂ than *Sacch. cerevisiae*.

Effects of growth phase on the induction of antioxidant enzymes

There are reports that bacterial cells and *Sacch. cerevisiae* acquire higher resistance to various stressful treatments as they proceed into stationary phase (Dowds *et al.*, 1987; Lange & Hengge-Aronis, 1991; Jamieson, 1992; Steels *et al.*, 1994). Induction of several antioxidant enzymes was observed to accompany this acquisition of resistance

Fig. 3. Survival rates of *Schiz. pombe* at different growth phases. Cells at early exponential (●), late exponential (▽) or stationary (▼) phase were treated with 40 mM H₂O₂ (a) or 6 mM MD (b). At 15 min intervals following treatment, aliquots were taken and plated on YES in duplicate. The survival of untreated cells was monitored as a control (○). Data are from one representative experiment.

(McCann *et al.*, 1991; Lowen *et al.*, 1985; Belázzi *et al.*, 1991). The survival of *Schiz. pombe* cells was measured at three different growth stages in YEPD medium following treatment with lethal concentrations of H₂O₂ or MD (Fig. 3). In order to exclude the possible effects of cell density on the survival rate, we diluted the cultured cells to the same turbidity at the time of oxidant treatment. An increase in resistance to both H₂O₂ and MD was observed as cells proceeded into stationary phase.

The levels of various antioxidant enzymes was also determined in cells at each growth stage, the results being summarized in Table 1. Catalase activity increased up to about 5-fold at stationary phase, which is higher than the amount induced by treatment of cells with H₂O₂ or MD. Both G6PD and GR increased up to almost 6-fold as growth proceeded to stationary phase. Peroxidase, on the

Table 2. Changes in intracellular concentrations of glutathione in *Schiz. pombe* at different growth phases, and in *Sacch. cerevisiae* at exponential phase

Cells were grown and cell-free extracts prepared as described in Methods. The values presented are means $\pm$ SD from 4 independent cultures.

Strains	Growth phase	Total glutathione concn [nmol (mg protein) ⁻¹]	GSH/GSSG ratio
<i>Schiz. pombe</i>	Early exponential	31.8 $\pm$ 1.2	22.5 $\pm$ 4.6
	Late exponential	32.0 $\pm$ 3.3	21.8 $\pm$ 4.4
	Stationary	35.5 $\pm$ 3.8	36.0 $\pm$ 4.3
<i>Sacch. cerevisiae</i>	Early exponential	29.5 $\pm$ 5.9	14.7 $\pm$ 2.8

other hand, increased only about 2-fold, whereas SOD remained unchanged as growth proceeded. The change in the level of catalase in our study is consistent with previous observations in *Sacch. cerevisiae* that catalase T is induced at the resting state when nutrients become limiting (Belázzi *et al.*, 1991). The insensitivity of SOD to change was further examined by differentiating cyanide-sensitive (possibly Cu/ZnSOD) and -insensitive (possibly MnSOD) activity. We found that cyanide-sensitive SOD, which constituted about 80% of the total SOD in early exponential phase, decreased to almost non-detectable levels as cells proceeded past late exponential phase. In contrast, cyanide-insensitive SOD increased about 6-fold, suggesting that MnSOD levels increase with respiratory adaptation as glucose is exhausted, as also occurs in *Sacch. cerevisiae* (Galiazzo & Labbe-Bois, 1933).

The increase of G6PD in the later stage of growth is somewhat surprising, since glucose is exhausted in the stationary phase. Studies with animal cells have demonstrated that fasting cells have lower levels of G6PD whereas refeeding increases its level (Kletzien *et al.*, 1994). In contrast, *Schiz. pombe* produced higher activities of G6PD under nutrient-limiting conditions. This may contribute to oxidative stress protection, since the G6PD reaction is a major generator of NADPH, providing the reducing equivalents for the regeneration of GSH by GR.

We also measured the level of intracellular glutathione at different growth phases (Table 2). The total amount of glutathione remained unchanged whereas the ratio of GSH to GSSG increased as cells proceeded into stationary phase. This increase in the steady-state level of reduced glutathione correlates with the marked increase in GR and G6PD activity and may contribute to the increased resistance of *Schiz. pombe* to oxidative stress in the stationary phase (Fig. 3). The higher level of GSH in *Schiz. pombe* compared to *Sacch. cerevisiae* may have also contributed to the greater resistance of *Schiz. pombe* to H₂O₂.

From the above results, we conclude that *Schiz. pombe* possesses adaptation mechanisms to counter oxidative

stress, involving the induction of several antioxidant enzymes. The superoxide-generating compound MD induced more antioxidant enzymes and to a greater extent than H₂O₂. The increase in resistance to oxidative stress at later growth phases also correlated well with the increase in these antioxidant enzymes. The involvement of other proteins in protecting *Schiz. pombe* against oxidation damage as observed in *Sacch. cerevisiae* needs to be studied further (Collinson & Dawes, 1992; Jamieson *et al.*, 1994).

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