Analysis of *Saccharomyces cerevisiae* proteins induced by peroxide and superoxide stress

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Exponentially growing *Saccharomyces cerevisiae* cells are more sensitive to oxidants such as hydrogen peroxide and superoxides than stationary phase cells. Using disruption mutations in the genes encoding the two *S. cerevisiae* superoxide dismutases, we show that the principal mechanism of toxicity of redox-cycling compounds, such as menadione and plumbagin, is via the production of superoxide anions. Using two-dimensional polyacrylamide gel electrophoresis we have compared the pattern of protein expression in cells labelled with L-[³⁵S]methionine and stressed with either H₂O₂ or menadione. Three groups of proteins were evident: those whose levels are elevated by both H₂O₂ and menadione, and those specifically induced by either H₂O₂ or menadione. Experiments with promoter fusions demonstrated that one of the heat inducible forms of HSP70 (*SSA1*) was inducible with H₂O₂. Furthermore, induction of the yeast H₂O₂-responsive *TRX2* promoter by menadione required the metabolism of menadione.

Keywords: yeast, oxidative stress, *Saccharomyces cerevisiae*

INTRODUCTION

All aerobically growing micro-organisms encounter reactive oxygen species (ROS), including the superoxide anion (O₂^{•−}), H₂O₂ and the hydroxyl radical. ROS can be generated as a consequence of normal cellular metabolic processes such as respiration or the β -oxidation of fatty acids. Alternatively, ROS can be produced through the metabolism of foreign chemicals or by the host immune system. Indeed, the microbiocidal effect of ROS is central to the destruction of potential pathogens (Baggiolini & Thelen, 1991). The toxicity of ROS is due to their ability to damage a large number of cellular constituents. For example, ROS produce double-stranded breaks and apurinic and apyrimidinic bases in DNA (Storz *et al.*, 1987). Damage to lipids by ROS arises from the formation of lipid peroxides, and, in proteins, amino acid side chains can become oxidized (Wolff *et al.*, 1986). As a result of the harmful nature of ROS, many aerobically growing organisms possess strategies for protection against oxidative stress, which involve the synthesis and activation of protective enzymes or molecules.

Considerable attention has been focused on the adaptive oxidative stress responses of bacteria, principally *Escherichia coli* and *Salmonella typhimurium*, although adaptive oxidative stress responses have been demonstrated in

Bacillus subtilis and *Streptomyces coelicolor* (Dowds *et al.*, 1987; Lee *et al.*, 1993). Treatment of *E. coli* with H₂O₂ results in the synthesis of about 30 proteins and causes the activation of a transcription factor (OxyR) which acts on the promoters of nine genes whose products are required for protection against oxidative stress (Storz *et al.*, 1990). There is also an additional stress response in *E. coli* to the presence of superoxide anions (Demple & Amabile-Cuevas, 1991) which is regulated by the *soxR* and *soxS* gene products and results in the induction of a number of genes including those encoding superoxide dismutase, glucose-6-phosphate dehydrogenase and the DNA repair enzyme exonuclease IV (reviewed by Demple, 1991). Despite the distinct regulatory nature of these adaptive stress responses there is some overlap between them, and several proteins are induced by both stress responses (Greenberg & Demple, 1989). Some of the overlap can be explained by the production of H₂O₂ from O₂^{•−} by both non-enzymic and enzymic mechanisms (Halliwell & Gutteridge, 1984).

The yeast *Saccharomyces cerevisiae* represents an ideal organism in which to study the regulation of gene expression in response to oxidative stress. This organism, unlike higher eukaryotes, is readily amenable to both genetic analysis and molecular biological techniques. Recent work has shown that *S. cerevisiae* possesses distinct inducible protective oxidative stress responses to both peroxides and superoxides (Jamieson, 1992; Collinson &

Abbreviations: ROS, reactive oxygen species; SOD, superoxide dismutase.

Dawes, 1992; Flattery-O'Brien *et al.*, 1993). This study demonstrates that the toxic action of the redox cycling compounds menadione and plumbagin is due to the production of superoxide anions. The profiles of proteins induced in response to both H_2O_2 and menadione have been compared by two-dimensional polyacrylamide gel electrophoresis, which shows that there are significant overlaps between the two stress responses. We have used gene fusions to a number of yeast promoters to demonstrate that they are regulated at the level of transcription by H_2O_2 .

METHODS

Yeast strains and techniques. Standard yeast methods and growth media were as described by Sherman *et al.* (1982). The yeast strains and plasmids used are described in Table 1. Disruption of the *SOD1* and *SOD2* genes was by the method of Rothstein (1983). Strains DJY118 and DJY119 were constructed by transformation, using lithium acetate (Ito *et al.*, 1983), with either a linearized *EcoRI/SalI* fragment of pDJ51, containing the *sod1::URA3* gene disruption, or an *EcoRI/HindIII* fragment of pDJ50, containing the *sod2::TRP1* gene disruption. To confirm that the *SOD1* and *SOD2* genes were disrupted, polymerase chain reactions (PCR) were performed on genomic DNA isolated from Ura⁺ and Trp⁺ transformants, respectively, using the primers described below. In all cases only the fragments corresponding to the disrupted genes were observed. The presence or absence of either the MnSOD or Cu/ZnSOD activity was checked by enzyme assays as described by McCord & Fridovich (1969). β -Galactosidase assays were performed as described by Rose *et al.* (1990). Menadione (bisulphite salt) and plumbagin were obtained from Sigma. H_2O_2 (30%, w/v) solution was obtained from BDH. Media

components were obtained from Difco. All other chemicals were of analytical grade.

Plasmid construction. The *sod1* insertion mutation was constructed by amplifying a 0.558 kb fragment of the *SOD1* gene, which includes most of the coding sequence, from genomic DNA using the following oligonucleotide primers in a PCR reaction: 5'-GCCGGTGTCTCTGGTG-3' and 5'-GGCCGC-CCCGGCACA-3'. The amplified fragment (from nucleotide +33 to +591) was cloned into pT7-Blue T (Novagen) then excised as an *EcoRI-SalI* fragment and ligated to the large *EcoRI-SalI* fragment of pBR322. The 1.116 kb *HindIII* fragment from YEP24, containing the *URA3* gene, was inserted into the *HindIII* site in the *SOD1* coding sequence, creating plasmid pDJ51. To create a deletion mutation in the *SOD2* gene the following oligonucleotide primers were used in a PCR reaction to amplify a 0.941 kb fragment containing the majority of the *SOD2* coding sequence from genomic DNA: 5'-TGTCATTGCTCTCCACCACAGC-3' and 5'-GGTTCTCTGCCTGGTGGTGAG-3'. The amplified fragment (from nucleotide +46 to +987) was subsequently cloned into pT7-Blue T (Novagen). The 0.88 kb *BglII* fragment from pFL45 (Bonneaud *et al.*, 1991), containing the *TRP1* gene was inserted between the two *BglII* sites internal to the *SOD2* gene, creating pDJ50. The positions of all PCR primers are given relative to the ATG codon.

***SOD1*, *SOD2*, *CTA1* and *CTT1::lacZ* gene fusions.** To construct the *SOD1*, *SOD2*, *CTA1* and *CTT1::lacZ* gene fusions the following oligonucleotide primer pairs, having either *XhoI* or *BamHI* sites at the 5' end, were used to amplify the promoter regions of these genes by PCR from genomic DNA. *SOD1*: 5'-CCGCTCGAGCTGCGACTCATCCATGTG-3', 5'-CGCGG-ATCCACTGCGACTGCTTGAAC-3'; *SOD2*: 5'-ACGCTC-GAGCGACGCCACCACCTATC-3', 5'-CGGGATCCGCG-AACATCCTGGTAC-3'; *CTA1*: 5'-CCGCTCCAGGAATT-CTTAGAAGGTGAAGAA-3', 5'-CGCGGATCCAATTTTC-GACATCTTCTAGGGT-3'; *CTT1*: 5'-CCGCTCCAGGGT-TGCAAGTCCGAGAAC-3', 5'-CGCGGATCCACGTTTCAT-TTGTAAGCTGA-3'.

The amplified DNA fragments contained the following regions of promoter sequence: *SOD1* from -412 to +20; *SOD2* from -551 to +11; *CTA1* from -820 to +11; *CTT1* from -588 to +14. The amplified promoter fragments were digested with *XhoI* and *BamHI* and inserted into the vector pLG670-Z (Guarente, 1983). Insertion of DNA into the *XhoI-BamHI* sites fuses the promoter fragment to the *E. coli lacZ* gene.

Menadione and H_2O_2 adaptation experiments. To determine the degree of protection against cell killing afforded by menadione and H_2O_2 pretreatment, exponential phase cultures of strain S150-2B or its isogenic *sod1* or *sod2* derivatives (DJY118 and DJY119, respectively) were grown aerobically in YPD (Sherman *et al.*, 1982) at 30 °C in the presence or absence of either H_2O_2 (0.4 mM) or menadione (1 mM) for 1 h. Cells were collected by centrifugation and washed once in distilled water prior to resuspension in an equal volume of fresh pre-warmed YPD medium. Aliquots (1 ml) of these cells were then incubated for 1 h at 30 °C with increasing levels of either menadione, plumbagin or H_2O_2 , after which time cells were washed once in distilled water, plated out at appropriate dilutions on YPD plates and incubated at 30 °C.

Two-dimensional polyacrylamide-gel electrophoresis. Cellular proteins were labelled prior to, or during exposure to, levels of oxidants sufficient to induce an adaptive response, either H_2O_2 (0.4 mM) or menadione (1 mM) by adding L-[³⁵S]methionine (1.85 MBq, 50 μ Ci; Amersham) to the growth

Table 1. Strains and plasmids

Strain or plasmid	Relevant genotype	Source or reference
<i>S. cerevisiae</i>		
S150-2B	<i>MATa ura3-52 trp1-289 his3-Δ1 leu2-3,112</i>	J. Beggs, ICMB, University of Edinburgh, UK
DJY118	<i>MATa ura3-52 trp1-289 his3-Δ1 leu2-3,112 sod1::URA3</i>	Derived from S150-2B
DJY119	<i>MATa ura3-52 trp1-289 his3-Δ1 leu2-3,112 sod2::TRP1</i>	Derived from S150-2B
Plasmids		
pDJ50	<i>sod2::TRP1</i>	This study
pDJ51	<i>sod1::URA3</i>	This study
pYDJ59	<i>SOD2::lacZ URA3</i>	This study
pYDJ60	<i>SOD1::lacZ URA3</i>	This study
pYDJ61	<i>CTA1::lacZ URA3</i>	This study
pYDJ64	<i>CTT1::lacZ URA3</i>	This study
pYTRX2	<i>TRX2::lacZ URA3</i>	Kuge & Jones (1994)
pZJHSE2-137	<i>SSA1::lacZ URA3</i>	Slater & Craig (1987)

medium (SD; Sherman *et al.*, 1982). Labelling was continued for 15 min, then the cells were pelleted and washed twice in 1 ml lysis buffer (50 mM Tris/HCl, pH 8.0; 150 mM NaCl; 1%, v/v, NP40). Cell pellets were resuspended in 100 μ l lysis buffer to which approximately 100 μ l glass beads were added (diameter 0.4 μ m; BDH). Cells were broken by vortexing for four 1 min cycles with 1 min on ice in between. After centrifugation to remove cell debris, 100 μ l of first dimension sample buffer (9 M urea; 2% NP40; 5%, v/v, β -mercaptoethanol; 1.6%, v/v, ampholyte pH 5–7; 0.4% ampholyte pH 3–10) was added to the extract. Equivalent amounts of radioactivity from each extract were loaded onto first dimension gels containing 9.2 M urea, 4% (v/v) acrylamide 2% NP40 1.6% ampholyte pH 5–7, 0.4% ampholyte pH 3–10, and subjected to electrophoresis at 750 V. Second dimension gels were SDS-PAGE gels (12% acrylamide) (Laemmli, 1970).

RESULTS AND DISCUSSION

Effect of growth phase on H_2O_2 and plumbagin sensitivity

It has been shown previously that *S. cerevisiae* cells from stationary phase cultures are considerably more resistant to oxidants than exponentially growing cultures (Jamieson, 1992). Further experiments revealed some variability in the resistance of different batch cultures towards oxidants. To characterize this in more detail we determined the resistance of cells to oxidants at various points in the growth of batch cultures. The results (Fig. 1) show that as cells in batch culture approach stationary phase, possibly at the point of diauxic shift, they become increasingly more resistant to both H_2O_2 and plumbagin. In all subsequent experiments we utilized cultures that were grown aerobically in YPD or SD medium and were in the early exponential phase of growth.

The toxicity of redox cyclers towards *S. cerevisiae* is due to the production of superoxide anions

It has been demonstrated previously that *S. cerevisiae* is sensitive both to H_2O_2 and to superoxide-generating agents such as menadione and plumbagin (Jamieson, 1992; Collinson & Dawes, 1992; Flattery-O'Brien *et al.*, 1993). To establish that cells were sensitive to superoxide-generating agents as a consequence of the production of superoxide anions, and not through some other means, we constructed a series of isogenic yeast strains that were disrupted in either the *SOD1* or the *SOD2* genes (encoding the Cu/ZnSOD and mitochondrial MnSOD respectively) (Birmingham-McDonogh *et al.*, 1988; Van Loon *et al.*, 1986). Strains DJY118 ($\Delta sod1::UR_{A3}$) and DJY119 ($\Delta sod2::TRP1$) were exposed to various concentrations of oxidants for 1 h and cell viability measured. The results (Fig. 2a) show that the $\Delta sod1$ mutant is 100-fold more sensitive to plumbagin than its isogenic parent. By contrast, the sensitivity of the $\Delta sod2$ strain to plumbagin is indistinguishable from that of the wild-type strain, S150-2B. The sensitivity of the $\Delta sod1$ and $\Delta sod2$ strains to H_2O_2 remains unchanged (Fig. 2b). These results provide strong evidence that the Cu/ZnSOD is the principal superoxide-dismutating activity under these growth conditions and that the toxic action of redox cyclers such as menadione

Fig. 1. Effect of growth in batch culture on oxidant sensitivity of yeast cells. Aliquots (1 ml) of batch cultures of S150-2B growing aerobically at 30 °C in YPD medium were withdrawn at various points in the growth cycle and exposed for 1 h to either 2.45 mM H_2O_2 (■) or 70 μ M plumbagin (□). Samples were then diluted and plated on to YPD plates to measure cell viability. Data are from a representative experiment.

Fig. 2. Sensitivity of SOD null mutants to redox-cycling compounds. Aliquots (1 ml) of aerobically grown, exponential phase cultures of S150-2B (□), DJY118 ($\Delta sod1$) (▲) and DJY119 ($\Delta sod2$) (●) were exposed to increasing concentrations of plumbagin (a) or H_2O_2 (b) for 1 h at 30 °C. Samples were then diluted and plated onto YPD plates to measure cell viability. Data are from a representative experiment.

Fig. 3. Two-dimensional gel analysis of protein extracts from yeast cells exposed to 0.4 mM H_2O_2 (b) or 1 mM menadione (c), and from control cells (a). Cells were exposed to the oxidants for 30 min and then labelled with L-[^{35}S]methionine for a further 15 min. Extracts were prepared as described in Methods. An equal amount of radioactivity was loaded onto each gel. Induced proteins are labelled by arrows and numbered.

and plumbagin is due to superoxide anion production. The role of the mitochondrially located MnSOD appears to be restricted to protecting cells against superoxide anions produced as by-products of respiration (Guidot *et al.*, 1993). The induction of the adaptive oxidative stress responses was examined in the Δsod mutants. The peroxide and superoxide adaptive stress responses were both functional in the *SOD2* deletion mutant (data not shown). In the *SOD1* deletion mutant, the peroxide stress response was normal; however, this strain was so sensitive to superoxide anion generators that no adaptive stress response could be measured by cell viability counts (data not shown).

Two-dimensional gel analysis of proteins induced by oxidants

To determine the number of proteins induced by both types of oxidative stress and the relationship between the two responses, labelled extracts from cells which had been exposed to oxidants were prepared. Cultures of exponentially growing yeast were exposed to oxidants for 30 min immediately prior to labelling proteins with [^{35}S]methionine; incubation with [^{35}S]methionine was carried out for 15 min at 30 °C and the labelled proteins were separated using SDS-PAGE (data not shown). To improve the resolution of individual proteins, labelled extracts were subjected to two-dimensional gel electrophoresis as described in Methods. The results (Fig. 3a, b, c) show the pattern of major proteins induced by the two stresses. Electrophoresis on two-dimensional gels allowed the resolution of 12 proteins whose levels are elevated in response to H_2O_2 and 18 proteins whose levels are increased by treatment with menadione. Analysis of the gels suggests that these proteins can be grouped, based on their pattern of expression: those induced solely by either H_2O_2 or menadione; and those induced by both oxidants (Table 2). Several of the proteins induced by both of the oxidants appear to correspond to previously identified heat-shock proteins (Petko & Lindquist, 1986) (for example, protein 10 corresponds to HSP26). This supports our previous finding that induction of the heat-shock response can provide cross-protection against both H_2O_2 and superoxides, raising the possibility that these heat-shock proteins may also protect against oxidative stress. Recently it has been demonstrated that HSP26 is also induced by osmotic stress (Varela *et al.*, 1992).

Regulation of gene expression by oxidant stress

Since some of the oxidant-induced proteins appear to correspond to heat-shock proteins, we looked at the expression of the yeast heat-shock gene *SSA1*, in the presence of oxidants. We utilized a gene fusion consisting of the *SSA1* promoter fused to the *E. coli lacZ* gene, which contained the sequences necessary for mediating the regulation of this gene in response to heat shock (Slater & Craig, 1987). Exponential phase cultures of strains carrying this gene fusion were exposed to levels of oxidants that elicit an adaptive response for 1 h and assayed for β -galactosidase activity. The results (Table 3)

Table 2. Proteins induced by exposure to H₂O₂ or menadione

Protein numbers correspond to those used in Fig. 3. Only those proteins that reproducibly showed elevated expression upon oxidant exposure are described.

H ₂ O ₂		Menadione	
Protein	Approx. mol. mass (kDa)	Protein	Approx. mol. mass (kDa)
1	156	1	156
2	97.4	3	77.8
3	77.8	5	72.9
4	72.9	13	61.3
5	72.9	14	53
6	41.7	6	41.7
7	40.4	8	36
8	36	15	30.4
9	32.3	16	28.4
10 (HSP26)	25.7	17	27.9
11	25	18	26.2
12 (HSP12)	14	19	25.7
		20	25.7
		10 (HSP26)	25.7
		21	25.5
		11	25
		22	21.7
		12 (HSP12)	14

Table 3. Effect of oxidants on transcription of the *SSA1*, *TRX2*, *SOD1*, *SOD2*, *CTA1*, *CTT1* genes

Cultures of S150-2B transformed with the gene fusion plasmids (Table 1) were grown aerobically at 30 °C in SD medium to early exponential phase (approximate OD₆₀₀ = 0.1). Cultures were then exposed to either H₂O₂ (0.4 mM) or menadione (1 mM) for 1 h. Cells were harvested and extracts prepared and assayed as described in Methods. Values presented are the means of duplicate assays ± range.

Gene fusion	Specific activity [nmol ONPG hydrolysed min ⁻¹ (mg protein) ⁻¹]		
	No stress	+ H ₂ O ₂	+ Menadione
<i>SSA1::lacZ</i>	4.85 ± 0.05	68.6 ± 1.6	5.45 ± 0.12
<i>TRX2::lacZ</i>	12.5 ± 0.07	286 ± 8.6	14.5 ± 0.35
<i>SOD1::lacZ</i>	1.673 ± 0.07	1.371 ± 0.01	1.838 ± 0.07
<i>SOD2::lacZ</i>	0.299 ± 0.03	0.473 ± 0.002	0.368 ± 0.03
<i>CTA1::lacZ</i>	0.159 ± 0.02	0.196 ± 0.01	0.126 ± 0.01
<i>CTT1::lacZ</i>	0.082 ± 0.006	0.178 ± 0.02	0.088 ± 0.02

demonstrate that this portion of the *SSA1* promoter is capable of increasing expression in response to H₂O₂ in addition to heat shock, showing that the levels of at least

one of the HSP70 isoforms of yeast is elevated by oxidative stress. Transcriptional regulation of the *SSA1* gene is similar to that of the yeast *TRX2* gene (encoding one of the two yeast thioredoxins), which has also recently been shown to be oxidant-inducible (Table 3) (Kuge & Jones, 1994).

We also constructed *lacZ* gene fusions to the *CTT1*, *CTA1*, *SOD1* and *SOD2* promoters, encoding the two yeast catalases and the mitochondrial MnSOD and cytosolic Cu/ZnSOD respectively, since these enzymes represent important defence systems against oxidants. Transformants of S150-2B containing the gene fusions were grown aerobically to early exponential phase and then treated for 1 h with either H₂O₂ or menadione to induce the adaptive responses. Extracts were prepared from these cultures and assayed for β-galactosidase activity. Neither superoxides nor H₂O₂ significantly affected the expression of either the MnSOD-encoding or the Cu/ZnSOD-encoding gene (Table 3). In agreement with an earlier report (Marchler *et al.*, 1993), transcription of the *CTT1* gene was shown to be inducible by H₂O₂, although the level of induction was low, only twofold (Table 3). Transcription of the *CTA1* gene was unaffected by exposure to oxidants. The lack of induction of the *SOD2* and *CTA1* genes in our assays is perhaps less surprising as cells grown in glucose express very little of the enzymes they encode because of glucose repression (Westerbeek-Marres *et al.*, 1988; Ruis & Hamilton, 1992).

The lack of inducibility of *SOD1* gene expression by oxidants was surprising. There are several possible explanations for this. It is possible that transcription of the *SOD1* gene is constitutive, as overexpression of SOD may be detrimental to cells; indeed, *E. coli* cells that overproduce the FeSOD are more susceptible to the toxic action of redox cyclers (Scott *et al.*, 1987). It is also possible that the important functional elements of the *SOD1* promoter lie further upstream of the DNA fragment used in the gene fusion construct. Another possibility is that aerobically grown cultures (as used in this study) already have maximally induced levels of the Cu/ZnSOD. These results suggest that although the Cu/ZnSOD is an important enzyme in countering the toxicity of superoxide anions, it is not an inducible part of the superoxide adaptive stress response.

Pretreatment of cells with menadione also induces the H₂O₂ adaptive stress response

The action of SOD results in the production of H₂O₂, and it therefore seemed likely that treatment of cells with 1 mM menadione could also lead to the induction of the H₂O₂ adaptive stress response as a result of the dismutation of superoxide anions. Indeed, previous work determined that treatment of yeast cells with menadione results in cross-protection against H₂O₂ (Jamieson, 1992). In order to test this idea we looked at the expression of the H₂O₂-responsive *TRX2* gene. Transcription of this gene has been shown to be induced by the presence of H₂O₂ (Kuge & Jones, 1994). Transformants of S150-2B carrying the *TRX2::lacZ* gene fusion were grown to

Fig. 4. Time course of induction of *TRX2* gene expression by oxidants. Cultures of S150-2B transformed with *pyTRX2::lacZ* were grown aerobically to early exponential phase ($OD_{600} = 0.1$) in SD medium and then exposed to either 0.4 mM H_2O_2 (▼) or 1 mM menadione (▽). At various time intervals after addition of oxidant aliquots of the cultures were assayed for β -galactosidase activity (Miller units) (Guarente, 1983). Values represent the means of duplicate experiments.

early exponential phase, cultures were treated with adaptive levels of either H_2O_2 or menadione and β -galactosidase activity was measured at various times after addition of the oxidants. Induction of *TRX2* gene expression by menadione proceeded more slowly than that observed with H_2O_2 , suggesting that exposure to menadione produces H_2O_2 by an indirect route (Fig. 4). In addition to the slower induction of expression, the maximal level of expression is lower than that seen after H_2O_2 treatment. This delay in expression is not observed with those proteins which can be induced by both H_2O_2 and menadione (Fig. 3; Table 2), which are clearly apparent within 1 h. This apparent discrepancy could be explained by the slower translation of the 116 kDa β -galactosidase protein (under oxidative stress conditions) compared to that of the 11.2 kDa *TRX2* protein. A more likely explanation would be to suggest that some proteins are genuinely induced by both types of oxidative stress, and possibly by other forms of stress, for example those encoding HSP12 and 26. Clarification of these ideas awaits identification and characterization of the induced proteins and their role in protection against stress.

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