Inducibility of the response of yeast cells to peroxide stress

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Exponential phase cells of the yeast *Saccharomyces cerevisiae* when treated with a non-lethal concentration of hydrogen peroxide (H_2O_2; 0·2 mM) for 60 min adapted to become resistant to the lethal effects of a higher dose of H_2O_2 (2 mM). From studies using cycloheximide to inhibit protein synthesis it appears that protein synthesis is required for maximal induction of resistance but that some degree of protection from the lethal effects of peroxide can be acquired in the absence of protein synthesis. Treatment of cells with 50 μg cycloheximide ml^{-1} alone led to them acquirng some protection from peroxide. Cells subjected to heat shock became more resistant to 2 mM-H_2O_2; however, peroxide pretreatment did not confer thermotolerance. L-[^{35}S]Methionine labelling of cells subjected to 0·2 mM-H_2O_2 stress showed that synthesis of at least ten polypeptides was induced by peroxide treatment. Some of these were also induced in cells subjected to heat shock (23 to 37 °C shift) but the synthesis of at least four polypeptides (45, 39·5, 38 and 24 kDa) was unique to peroxide-stressed cells. Resistance to peroxide was also inducible in an isogenic petite and an isogenic strain with a mutation in the *HAP1* gene, indicating that the adaptive response does not require functional mitochondria.

Introduction

Reactive oxygen species are well known toxic agents capable of killing cells rapidly. The reactive species hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and the hydroxyl radical ($^{\cdot}OH$) can damage a variety of cellular components causing lipid peroxidation, oxidation of proteins and DNA lesions (Dean & Simpson, 1989; Imlay *et al.*, 1988). The effects of these oxidants have been implicated in cancer, cardiovascular disease and ageing; recently, they have also been shown to enhance the expression and replication of HIV-1 (Schreck *et al.*, 1991). Biological systems have evolved several defence mechanisms which enable cells to cope with lethal oxidative environments. These antioxidant defence systems include enzymic activities such as superoxide dismutase and catalase that detoxify the oxidants, and non-enzymic protective molecules including glutathione, vitamins C and E and uric acid (Scandalios, 1990).

The response of cells to oxidative stress has been most extensively studied in the prokaryotes *Escherichia coli* and *Salmonella typhimurium*. When these bacteria are exposed to a low, non-lethal dose of oxidant they adapt to become resistant to subsequent lethal challenges. The observed adaptive response requires the induction of protein synthesis since if exposed to low doses in the presence of protein synthesis inhibitors these cells no longer acquire resistance to lethal oxidant doses (Christman *et al.*, 1985). Many of the proteins and their corresponding genes involved in bacterial defences against oxidative stress have been identified (Storz *et al.*, 1990). In addition to the induction of proteins directly involved in detoxifying and repairing damage by oxidants and free radical species, some of the inducible proteins overlap with heat-shock-inducible polypeptides. For example, the *S. typhimurium* H_2O_2-resistant mutant *oxyR1* selected under oxidative conditions constitutively overexpresses five heat shock proteins (Morgan *et al.*, 1986).

The oxidative stress response and its relationship to the heat shock phenomena is also being intensely investigated in the eukaryotic systems *Drosophila melanogaster*, the yeast *Saccharomyces cerevisiae* and in maize. Much of the work has focussed on the identification and isolation of genes involved in scavenging free radicals, particularly the superoxide dismutase and catalase genes (Scandalios, 1990; Phillips & Hilliker, 1990; Cross & Ruis, 1978). Unlike the bacterial systems described earlier, little is known about how eukaryotic cells co-ordinate gene expression in response to oxidative

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stress (Storz *et al.*, 1990). The yeast *Saccharomyces cerevisiae* is an ideal organism to study the co-ordination of cellular defences against oxidative stress since its microbial habit facilitates physiological studies of the effects of oxidants and the selection of mutants which are altered in their response to them.

Here the nature of the response of *S. cerevisiae* cells to peroxide stress was examined in terms of the effects of peroxide concentration, the inducibility of the response of cells to peroxide, the relationship between the heat shock response and the response to peroxide treatment. The effects on protein synthesis in cells subjected to each of the forms of stress (heat and peroxide) has been examined. The role of the mitochondria as a primary *in vivo* source of free radicals in the adaptation of yeast cells to peroxide stress has also been examined.

Methods

Yeast strains and media. The wild-type yeast strain BGW1-7a (*MAT***a** *ade1-100 his4-519 leu2-2,2-112 ura3-52*) and the isogenic *hap1* disruption mutant LYP22 were kindly supplied by Leonard Guarente (Massachusetts Institute of Technology, Cambridge, MA, USA). The respiratory incompetent (petite) strain Y3P was obtained by treating BWG1-7a with ethidium bromide (Spencer & Spencer, 1988). Yeast cultures (50 ml) were routinely grown in YEPD medium containing 2% (w/v) glucose, 2% (w/v) bactopeptone, 1% (w/v) yeast extract at 30 °C with shaking to an OD_{600} of 0·1. This represented about $1{\cdot}8 \times 10^6$ viable cells ml^{-1} prior to peroxide treatment (taken as 100% survival value).

Peroxide treatment and heat shock conditions. Cells were harvested by centrifugation at 4000 *g* for 5 min and resuspended in 100 mM-potassium phosphate buffer, pH 7·4. A dose response curve was determined by treating 5 ml samples with various concentrations of H_2O_2 added from a 30% (w/v) stock solution. For adaptation experiments, cells were harvested, resuspended in fresh YEPD media to which H_2O_2 was added (0·2 mM) and incubated with shaking at 30 °C for 60 min. Pretreated cells were harvested by centrifugation, resuspended in 100 mM-phosphate buffer, pH 7·4, and challenged with 2 mM-H_2O_2.

Cell survival was monitored by taking samples at 15 min intervals, diluting in 100 mM-phosphate buffer (pH 7·4) and plating aliquots on YEPD plates. To inhibit cytoplasmic protein synthesis, cycloheximide (50 μg ml^{-1}) was added during the peroxide adaptation and challenge periods. Cultures grown at 23 °C with shaking were exposed to a mild heat shock by incubation at 37 °C for 60 min. Heat stress was induced by incubating cultures at 52 °C. Samples were taken at 2 min intervals, diluted in phosphate buffer and plated on YEPD to obtain viable cell counts. All plate counts were done in duplicate, and experiments were repeated at least three times.

L-[^{35}S]Methionine labelling of cellular proteins. Cells were grown in minimal glucose medium (per litre: 20 g glucose, 1·7 g Difco yeast nitrogen base, 5 g $(NH_4)_2SO_4$, 40 mg auxotrophic requirements) supplemented with 30 mg L-tyrosine l^{-1}. Cultures grown at 23 °C to an OD_{600} of 0·1 were harvested and resuspended in fresh minimal glucose medium. Samples (10 ml) were exposed to either no stress, or oxidative (0·2 mM-H_2O_2) stress or heat stress (37 °C) conditions and at 5 min intervals were pulse-labelled (10 min) with 25 μCi (0·93 MBq) L-[^{35}S]methionine (Trans ^{35}S-Label, ICN Biochemicals). Incorporation of the radiolabelled amino acid was terminated by the addition of cycloheximide (100 μg ml^{-1}) and rapid cooling on ice. Cells were pelleted and resuspended in 300 μl of SDS-lysis buffer (0·0625 M-Tris/HCl, pH 6·8, 5%, v/v, 2-mercaptoethanol, 3%, w/v, SDS, 4 mM-phenylmethylsulphonyl fluoride) plus 10 μl of a solution of 0·5 mg RNAase/1 mg DNAase ml^{-1}. To disrupt cells, glass beads were added (0·3 g) and the microfuge tubes were vortexed for 2 min. Proteins were precipitated with 10% (w/v) trichloroacetic acid (TCA), washed with an ether/ethanol (1:1, v/v) solution, dried *in vacuo* and resuspended in SDS buffer. Uptake studies were performed by adding 10 μCi (370 kBq) L-[^{35}S]methionine to 5 ml cultures of BWG1-7a grown in glucose minimal media in the presence or absence of cycloheximide (50 μg ml^{-1}). At 5 min intervals 200 μl samples were taken, precipitated onto glassfibre discs with 10% (w/v) TCA and washed with 10 ml of cold methionine/cysteine solution (each at 100 μg ml^{-1}). Levels of incorporation were determined by counting in toluene-based scintillation fluid using a Tri-Carb 1900 scintillation counter.

SDS-PAGE. Protein samples containing 10% (v/v) glycerol and 0·001% bromophenol blue were boiled for 2 min prior to electrophoresis. A 10 to 15% (w/v) gradient polyacrylamide-SDS slab gel was used with the discontinuous buffer system of Laemmli (1970). Gels were electrophoresed at room temperature at 30 mA for 270 v h. Gels were fixed for 60 min in a 40% (v/v) methanol, 10% (v/v) acetic acid bath and dried using a Bio-Rad gel dryer. Dry gels were autoradiographed using pre-flashed FujiRX X-ray film at −70 °C for 6 d.

Results and Discussion

Sensitivity of yeast cells to H_2O_2

To determine the concentrations of H_2O_2 affecting the yeast strain BGW1-7a, a dose response curve was determined by treating exponentially growing yeast cells with various concentrations of H_2O_2 (Fig. 1). The lethal effect of peroxide on cultured yeast cells is apparently dependent on cell density since stationary phase cultures are more sensitive to higher concentrations, in the order of 10 to 20 mM (Steels *et al.*, 1991), when compared to early exponential phase cells, as reported here. This is interesting since cells entering stationary phase become more resistant to other forms of stress, including heat shock (Schenberg-Frascino & Moustacchi, 1972). Early exponential phase cultures used for this investigation were very sensitive to peroxide concentrations greater than 1 mM. In subsequent experiments an H_2O_2 concentration of 2 mM was chosen to test for lethality and cells were exposed to 0·2 mM-H_2O_2 for 1 h as a non-lethal treatment.

Adaptation of yeast cells to H_2O_2

Exponentially growing yeast cells were treated with a non-lethal dose of H_2O_2 (0·2 mM) in glucose-containing complete medium (YEPD) for 60 min at 30 °C. At the end of the pretreatment period cells were harvested and challenged with a lethal H_2O_2 concentration (2 mM) in

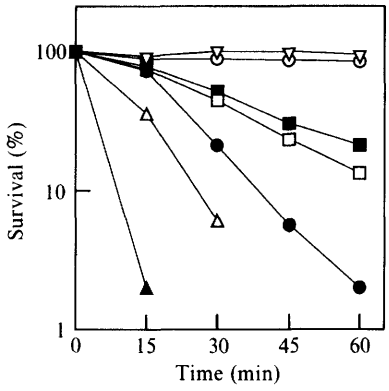

Fig. 1

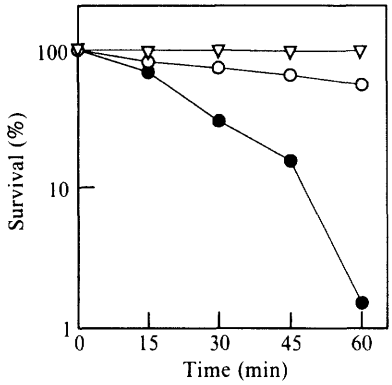

Fig. 2

Fig. 1. Sensitivity of yeast cells to H_2O_2. Cells of strain BWG1-7a were grown exponentially at 30 °C to an OD_{600} of 0·1. This represented $1·8 \times 10^6$ cells ml^{-1} which is the 100% survival value. Cells were treated with various concentrations of H_2O_2 for 60 min. Samples were diluted and plated on YEPD solid media to monitor cell viability. An H_2O_2 concentration of 2 mM was chosen as lethal dose for subsequent experiments. H_2O_2 concentrations (mM) were: 0·1 (○); 0·5 (■); 1 (□); 2 (●); 4 (△); 10 (▲). ▽, Control. Data are the means of duplicates from a representative experiment.

Fig. 2. Induction of peroxide tolerance. Yeast cells were treated with 0·2 mM-H_2O_2 for 60 min then challenged with a lethal (2 mM) dose of H_2O_2 (○). ●, 2 mM-treated cells without pretreatment; ▽, control. Samples were diluted and plated on YEPD to monitor cell viability. The data are from a representative experiment.

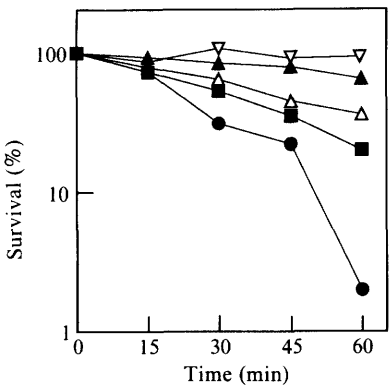

Fig. 3

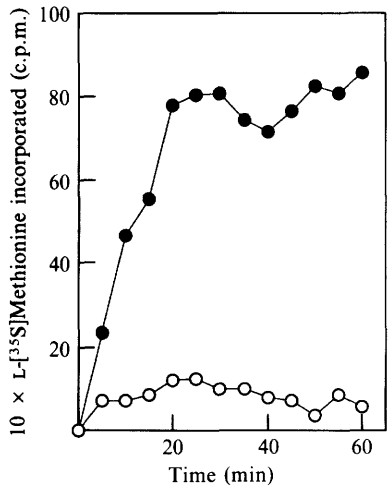

Fig. 4

Fig. 3. Effect of cycloheximide on the acquisition of peroxide resistance. Yeast cells were incubated with cycloheximide (50 μg ml^{-1}) during the pretreatment period followed by a challenge with 2 mM-H_2O_2 (■). ▽, Control; ▲, 0·2 mM-H_2O_2 pretreatment + 2 mM-H_2O_2 challenge; △, cycloheximide pretreatment only + 2 mM-H_2O_2 challenge; ●, 2 mM-H_2O_2 treated. Samples were diluted and plated on YEPD to monitor cell viability. The data are from a representative experiment.

Fig. 4. Cycloheximide effectively inhibits amino acid incorporation. Yeast cells were grown in glucose minimal media supplemented with L-tyrosine (30 mg l^{-1}). The rate of L-[^{35}S]methionine incorporation into TCA-precipitable material was followed in the presence (○) or absence (●) of cycloheximide (50 μg ml^{-1}). Data are the means of duplicates from a representative experiment.

potassium phosphate buffer. From Fig. 2 it can be seen that cells pretreated with 0·2 mM-H_2O_2 were much more resistant to killing by 2 mM-H_2O_2 than those in untreated control cultures. This establishes that yeast cells can adapt to a non-lethal treatment with peroxide, and it raises the question of whether this adaptation depends on a modification of existing components of the cells or on the synthesis of proteins during the adaptation phase.

Effect of cycloheximide on the acquisition of peroxide resistance

To determine whether protein synthesis is required for peroxide-induced resistance to 2 mM-H_2O_2, cycloheximide was added as an inhibitor of cytoplasmic protein synthesis during the peroxide adaptation phase. The presence of cycloheximide led to a partial reduction

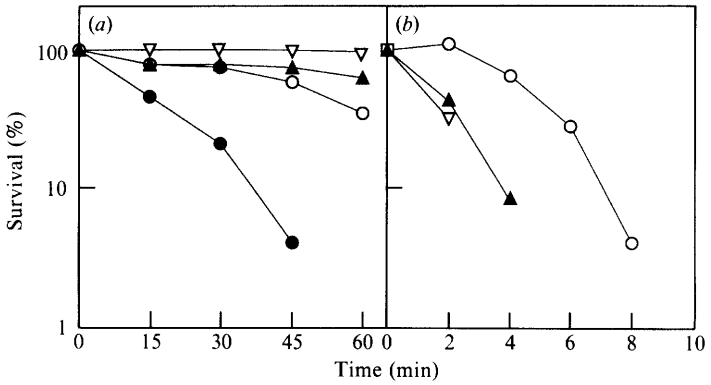

Fig. 5. Induction of peroxide resistance does not confer thermotolerance. Yeast cells, grown at 23 °C, were heat-shocked (37 °C; ○) or pretreated with H_2O_2 (0·2 mM; ▲) followed by a challenge (*a*) with 2 mM-H_2O_2 or (*b*) by incubation at 52 °C. ▽, Control; ●, 2 mM treatment only. Samples were taken, diluted and plated on YEPD to monitor cell viability. Where time points are not given survival was <1% of the control. The data are from a representative experiment.

in the ability of the cells to survive 2 mM-H_2O_2 (Fig. 3), indicating that at least part of the adaptive response may depend on protein synthesis. Belazzi *et al.* (1991) have observed that cycloheximide alone, present in the culture medium, induces an increase in yeast catalase T mRNA transcript levels. An important control was done in which cells preincubated with cycloheximide for 60 min were challenged with 2 mM-H_2O_2. Surprisingly, cycloheximide-pretreated cells were not as sensitive to 2 mM-H_2O_2 when compared to untreated cells. [^{35}S]Methionine incorporation studies were performed to confirm that the concentration of cycloheximide used during the peroxide exposure phase was effectively inhibiting amino acid incorporation into TCA-precipitable material (Fig. 4). This result using cycloheximide makes it difficult to assess the role of protein synthesis on the induction of peroxide resistance. Cells pretreated with both cycloheximide and 0·2 mM-H_2O_2 were less resistant than those treated with either agent alone, which does indicate some protein synthesis is required for acquisition of true resistance. However, it is clear from the data that some degree of protection from the lethal effects of peroxide can be acquired in the absence of protein synthesis.

Induction of peroxide resistance does not induce thermotolerance

K. Watson and co-workers have shown that yeast cells subjected to a mild heat shock acquire tolerance to oxidative damage induced by H_2O_2 (Watson, 1990). The resistance to peroxide stress induced by heat shock (37 °C) is not as extensive as that in cells pretreated with H_2O_2 (Fig. 5). A characteristic of the heat shock response in yeast is that treated cells become much more resistant to thermal injury (assessed at 52 °C) than untreated cells (Watson, 1987). This raises the question of the nature of the relationship between the two types of stress response, and whether peroxide stress induces any degree of thermotolerance.

Exponential phase cultures were subjected to either a heat shock (by transfer from 23 to 37 °C for 60 min) or peroxide stress (0·2 mM-H_2O_2 for 60 min) or to neither form of stress and were then assessed for the effect of the pretreatment on the ability to survive 2 mM-H_2O_2 (Fig. 5*a*) or 52 °C (Fig. 5*b*). Some degree of resistance to peroxide was induced by this heat shock but this was not as extensive as that of cells pretreated with 0·2 mM-H_2O_2. However, pretreatment of cells with peroxide did not induce a significant degree of thermotolerance.

Polypeptide synthesis during peroxide stress

From the above experiments using cycloheximide it appeared that there may be some protein synthesis required during the adaptation to peroxide treatment for full protection to be achieved. This leads to the question of whether there are any polypeptides synthesized during peroxide stress, and if so whether any of these are produced by heat stress or are unique to peroxide stress.

[^{35}S]Methionine pulse-labelling experiments were performed to determine whether there are any changes in the pattern of synthesis of cellular polypeptides during peroxide stress. Cells grown in minimal glucose medium (23 °C) were subjected to either no stress, peroxide stress (0·2 mM-H_2O_2) or heat shock (37 °C) and pulse-labelled at zero time and at 5 min intervals up to 30 min.

Fig. 6 illustrates a one-dimensional SDS-polyacrylamide gel showing separation of polypeptides synthesized at different times after imposition of stress conditions. Even with the limited resolution of one-dimensional analysis, a number of observations can be

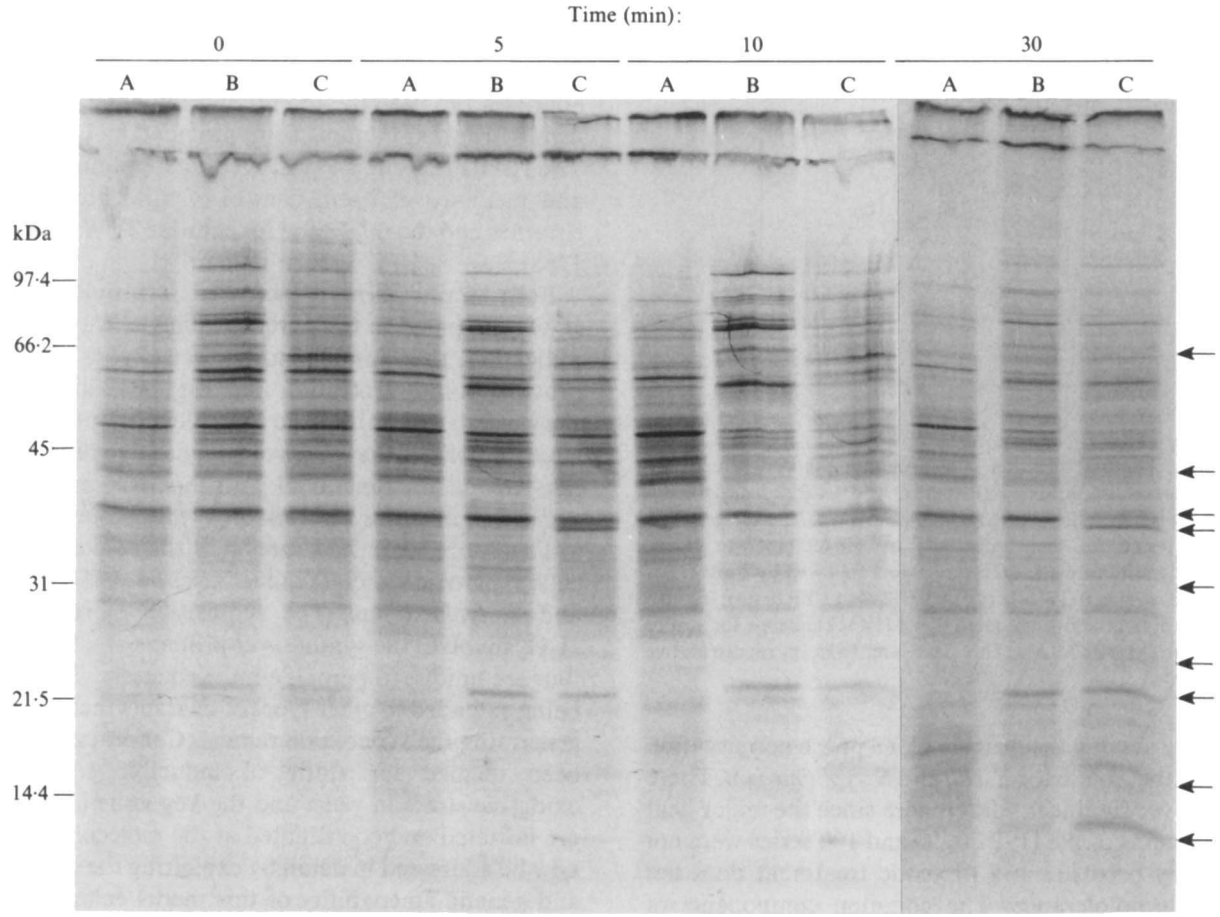

Fig. 6. Polypeptides synthesized during peroxide adaptation and a mild heat shock. Yeast cells were grown in glucose minimal media supplemented with L-tyrosine (30 mg l^{-1}). At the times indicated cultures were pulse-labelled (10 min) with 30 μCi L-[^{35}S]methionine. Conditions used were: (A) control, (B) heat shock (37 °C) and (C) peroxide treatment (0·2 mM). Peroxide-inducible polypeptides are indicated by arrows and are discussed in the text. Protein standards used were: rabbit muscle phosphorylase B (97·4 kDa); bovine serum albumin (66·2 kDa); ovalbumin (45 kDa); bovine carbonic anhydrase (31 kDa); soy-bean trypsin inhibitor (21·5 kDa) and hen egg lysozyme (14·4 kDa).

made. Within 30 min of imposing peroxide stress at least 10 polypeptides were synthesized that were not made in untreated cells. Prominent amongst these were polypeptides of 13, 16, 22, 38 and 65 kDa, all of which appeared within 10 min of addition of peroxide. Of the detectable changes, four (45, 39·5, 38 and 24 kDa) were induced by peroxide treatment but did not appear in either the control or heat-shocked cultures, indicating the existence of a set of unique peroxide-inducible proteins.

Some other changes were also induced by heat shock (e.g. the 13, 22 and 65 kDa polypeptides) but two of these (13 and 65 kDa) were more strongly induced by 0·2 mM-H_2O_2 treatment. It is interesting to note that while peroxide treatment did induce a subset of the heat shock polypeptides this did not include the major heat shock inducible proteins (HSPs) of the HSP 70, 90 and 100 groups that were clearly induced by the heat shock used in these experiments.

In *Drosophila*, treatment of cells with 1 mM-H_2O_2 leads to a 2·5-fold increase in the synthesis of HSP 70-68 and HSP 23 (Courgeon *et al.*, 1988). However, the appearance of HSP 23 was only detectable by two-dimensional electrophoresis while induction of HSP 70-68 was maximal after 2 to 3 h incubation with peroxide. Interestingly, transcription of the HSP 70-68 genes occurred within 10 min, well before translation. To ascertain whether activation of the major HSP genes occurs during peroxide stress may therefore require analysis of transcription and a longer incubation period, although the difference in yeast in induction of the proteins following heat shock and peroxide stress is clearly evident in Fig. 6. For *S. typhimurium*, two-dimensional gel electrophoresis revealed that H_2O_2 induced the synthesis of at least 30 proteins (Morgan *et al.*, 1986), and work is under way to increase the resolution of our results with yeast by this technique.

Both the physiological and pulse-labelling studies indicate that the response of yeast cells to peroxide stress

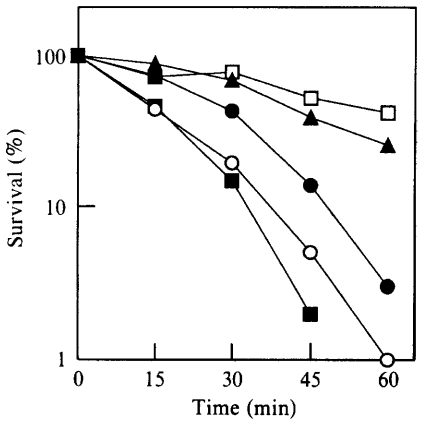

Fig. 7. Mitochondrial function is not an absolute requirement for peroxide adaptation. Strains Y3P (petite) and LYP22 (*hap1* disruption), otherwise isogenic with the wild type BGW1-7a, were grown at 30 °C and tested for their sensitivity and inducibility to H_2O_2. Incubation conditions were: no pretreatment followed by 2 mM-H_2O_2 treatment for strains BGW1-7a (●), Y3P (■) and LYP22 (○); 0·2 mM-H_2O_2 pretreatment and subsequent 2 mM-H_2O_2 challenge for strains Y3P (□) and LYP22 (▲). The data are from a representative experiment.

may partly overlap the heat shock response in a situation similar to that found for *E. coli* and *S. typhimurium*. There are, however significant differences since the major heat shock proteins of the HSP 70, 90 and 100 series were not induced by peroxide and peroxide treatment does not confer thermotolerance. The common components of these stress defence mechanisms may depend on the induction of similar proteins in conjunction with the modification of existing cellular components. For example, this could occur by phosphorylation-dependent activation of constitutively expressed stress proteins as proposed for activation of the yeast heat shock regulatory factor (Sorger & Pelham, 1988).

Is mitochondrial function required for peroxide resistance?

The mitochondrion is one source of free radical production in the cell, for example by the incomplete reduction of molecular oxygen leading to formation of the superoxide anion (O_2^-) and H_2O_2. Catalase, an extramitochondrial enzyme, and the mitochondrial superoxide dismutase are involved in detoxifying free radicals. Winkler *et al.* (1988) have suggested that synthesis of mitochondrial cytochromes and the haemoprotein catalase is coordinated at transcription by the HAP1 regulatory protein. This coordinated control of synthesis may be necessary for peroxide tolerance as feedback mechanisms may operate to activate nuclear transcription of genes encoding antioxidant enzymes.

To determine whether functional mitochondria are important for the adaptive response to peroxide, this response was tested in two mutant yeast strains. The first as a ρ^0 petite (strain Y3P) generated from BGW1-7a by ethidium bromide treatment, and the other an isogenic *hap1* disruption mutant strain provided by L. Guarente. The HAP1 protein is a positive transcriptional activator and mediator of haem control of mitochondrial cytochromes and the *CTT* gene for catalase T (Winkler *et al.*, 1988).

Both strains showed increased sensitivity to 2 mM-H_2O_2 compared to the parent strain BGW1-7a (Fig. 7), indicating that mitochondrial activity is needed to provide some protection against peroxide damage. However, both strains were still able to adapt to peroxide stress indicating that much of the adaptive response does not depend on functional mitochondria or on HAP1 activation of genes responsive to this protein.

The above work illustrates that the response of yeast cells to peroxide stress is inducible as described for *E. coli* and *S. typhimurium*. The acquisition of resistance to H_2O_2 involves the synthesis of proteins of which at least four are unique to peroxide-induced stress. This work is being extended to other types of oxidants including those generating the superoxide radical. Conditions have now been defined for study of inducible responses to oxidative stress in yeast and the key issue of how these are initiated and coordinated at the molecular level can now be addressed in detail by exploiting the biochemical and genetic amenability of this model eukaryote.

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