Hypoxia abolishes transience of the heat-shock response in the methylotrophic yeast Hansenula polymorpha

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The heat-shock response is conserved amongst practically all organisms. Almost invariably, the massive heat-shock protein (Hsp) synthesis that it induces is subsequently down-regulated, making this a transient, not a sustained, stress response. This study investigated whether the heat-shock response displays any unusual features in the methylotrophic yeast Hansenula polymorpha, since this organism exhibits the highest growth temperature (49–50 °C) identified to date for any yeast and grows at 47 °C without either thermal death or detriment to final biomass yield. Maximal levels of Hsp induction were observed with a temperature upshift of H. polymorpha from 30 °C to 47–49 °C. This heat shock induces a prolonged growth arrest, heat-shock protein synthesis being down-regulated long before growth resumes at such high temperatures. A 30 °C to 49 °C heat shock also induced thermotolerance, although H. polymorpha cells in balanced growth at 49 °C were intrinsically thermotolerant. Unexpectedly, the normal transience of the H. polymorpha heat-shock response was suppressed completely by imposing the additional stress of hypoxia at the time of the 30 °C to 49 °C temperature upshift. Hypoxia abolishing the transience of the heat-shock response appears to operate at the level of Hsp gene transcription, since the heat-induced Hsp70 mRNA was transiently induced in a heat-shocked normoxic culture but displayed sustained induction in a culture deprived of oxygen at the time of temperature upshift.

INTRODUCTION

Hansenula polymorpha (syn. Pichia angusta) appears to be the most thermotolerant yeast identified to date, its maximal growth temperature (49–50 °C; Cabeca-Silva & Madiera-Lopes, 1984; van Uden, 1984) being only about 10 °C lower than the apparent upper temperature limits for the growth for eukaryotic organisms in general (Maheshwari et al., 2000; Sechbach, 1994). H. polymorpha is also of interest as a production host for recombinant proteins and as one of the few yeasts capable of growth on methanol as a sole carbon source (reviewed by Gellissen, 2000; Hollenberg & Gellissen, 1997; Sudbery, 1996; van Dijk et al., 2000). Major advantages of this organism as an expression system include the availability of strong tightly regulated promoters and a high secretory capability, usually without the hyperglycosylation of secreted products so often encountered with Saccharomyces cerevisiae. In addition, an absence of catabolite repression of respiratory growth leads to the efficient conversion of high-sugar substrates to H. polymorpha biomass in simple batch fermentations. H. polymorpha achieves optimal growth rates at temperatures around 38–45 °C, yet grows at up to 47 °C with no thermal death and without any detriment to biomass yield (Cabeca-Silva & Madiera-Lopes, 1984; van Uden, 1984). This allows bioprocesses involving H. polymorpha to be operated at high temperature, reducing both the risk of bacterial contamination and the need for substantial cooling of large fermenter cultures.

In almost all living systems, temperature upshift elicits the heat-shock response. This response leads to strong induction of a conserved set of proteins (the heat-shock proteins, Hsps), and suppression of synthesis of most of the proteins made prior to temperature upshift (reviewed by Morimoto et al., 1997; Parsell & Lindquist, 1993; Piper, 1993). Generally, the strong Hsp synthesis that is triggered by an upshift to slightly supraoptimal, but sublethal, temperatures is subsequently down-regulated. When the cells are maintained
at such high temperatures, the synthesis of Hsps is eventually seen to decline, levelling off at lower basal levels that are still higher than the levels of Hsp synthesis that were present before the heat shock. The heat-shock response is therefore a transient, not a sustained stress response.

The heat-shock response has been subjected to very extensive molecular genetic analysis both in the bacterium Escherichia coli (Ades et al., 1999; Craig & Gross, 1991) and in the yeast S. cerevisiae (Lindquist & Kim, 1996; Parsell & Lindquist, 1993; Piper, 1993). These studies have established the importance of some of the induced Hsps for the capacity of these organisms to grow at high temperatures and to survive higher, even more extreme temperatures (i.e. to acquire thermotolerance) (Craig & Gross, 1991; Lindquist & Kim, 1996; Morimoto et al., 1997; Parsell & Lindquist, 1993; Piper, 1993). Some Hsps exert their protective effects by preventing and/or repairing partial protein denaturation at stressful temperatures, by binding to such proteins so to prevent their aggregation, by eliminating them through selective proteolysis, or by assisting in their refolding (Ellis & van der Vries, 1991; Gething & Sambrook, 1992; Lindquist & Kim, 1996; Parsell & Lindquist, 1993; Welch, 1991). Hsps also provide several essential functions during normal growth, many of them providing vital chaperone functions that help to catalyse protein folding and the translocation of protein precursors across membranes (Morimoto et al., 1997).

In this study, we investigated whether or not H. polymorpha, as the yeast with the highest growth temperature identified to date, displays any unusual features of its heat-shock response. A few studies had already been published on the individual Hsps of H. polymorpha (Titonenko et al., 1996; Tsiomenko et al., 1997), but not on the overall patterns of Hsp expression in this yeast. Unexpectedly, we found the normal transience of the Hsp expression in this yeast. Unexpectedly, we found the normal transience of the heat-shock response. A few studies had already been published on the individual Hsps of H. polymorpha (Titonenko et al., 1996; Tsiomenko et al., 1997), but not on the overall patterns of Hsp expression in this yeast. Unexpectedly, we found the normal transience of the H. polymorpha heat-shock response to be suppressed by hypoxia. As far as we are aware, this is the first study showing such a pronounced effect of oxygen levels on the heat-shock response.

**METHODS**

**Strains and growth conditions.** H. polymorpha strain NCYC 495 was used throughout. Growth media were: YPD (2 % w/v, glucose, 2 % peptone, 1 % yeast extract); YPM (1 %, v/v, methanol, 2 % peptone, 1 % yeast extract) and, for pulse-labelling experiments, minimal SD (2 % glucose, 0-67 % yeast nitrogen base without amino acids (Difco)) and SM (1 % methanol, 0-67 % yeast nitrogen base without amino acids). Cultures were initially grown at the indicated temperatures in shake-flask cultures under normoxic conditions; the volume of liquid occupied no more than 15 % of the flask volume.

**Heat shock, pulse-labelling and measurement of oxygen levels.** YPD and YPM cultures were grown to mid-exponential phase (1–2 x 10^8 cells ml^-1) at 30 °C, 37 °C and 49 °C, washed twice in water, and resuspended in 30 °C or 37 °C SD or SM to a density of 1 x 10^8 cells ml^-1. One hour later, they were divided into 20 ml aliquots and transferred to prewarmed flasks in a shaking waterbath set at the heat-shock temperature. One aliquot was maintained at the initial temperature as control. With all temperature-shift experiments, the cultures reached their final temperature in less than 3 min.

**In vivo labelling** was initiated by the addition of 1–4,5-^3^H-leucine [85 Ci mM^-1 (Amersham) to 3 μCi ml^-1 final concentration] at the indicated times before or after temperature shift. Labelling was terminated by a rapid addition of 30 μg ml^-1 of non-radioactive leucine and the dilution of the samples with 1 volume of ice-cold water. Cells were collected by centrifugation, washed once with water, and either processed immediately or quickly frozen and stored at –80 °C.

Partial anoxia was imposed at the time of heat shock by the transfer of normoxic 30 °C cultures to a sealed vessel, so that the medium now occupied 90 % of the tube volume. A 4 ml stirred jacketed vessel, with in-built oxygen electrode, was found convenient for measuring the depletion of dissolved oxygen by these heat-shocked actively respiring cells. This apparatus, originally constructed for rat heart perfusion experiments, was kindly loaned by Iain Mowbray. For our purposes, it was maintained at 49 °C, the cells being transferred to the stirred chamber at time zero in the heat-shock experiment.

**Preparation of crude extracts and SDS-PAGE analyses.** Total protein of H. polymorpha was extracted and analysed for pulse-labelled proteins on 12-5 % SDS gels, exactly as described previously for analyses of Hsps in S. cerevisiae (Cheng & Piper, 1994; Panaretou & Piper, 1992; Piper et al., 1994).

**Hsp70 mRNA analysis.** Samples of total RNA were prepared, Northern blotted and hybridized to an HpHSA1 gene (Titorenko et al., 1996) probe fragment, as previously described (Piper et al., 1998). The probe fragment was PCR amplified from H polymorpha genomic DNA, using primers 5’-ATGCTCTAAACGTGGGAATGGC-3’ and 5’-TCCACCTCTTGGACGGAAG-3’. Mapping of the 5’ ends of HpHSA1 transcripts was by AMV reverse transcriptase-catalysed primer extension (Ausubel et al., 1995), using a [5’-^32^P] end-labelled primer (TCTGTACCTTGCGATTG) that anneals at +82 to +103 on the HpHSA1 ORF. DNA sequencing reactions, run in parallel on the same gel, were prepared using the same primer and plasmid pHSA1 (Titorenko et al., 1996) as template.

**Thermotolerance experiments.** Mid-exponential cultures were grown at the indicated temperatures for at least five generations, or heat shocked as described, then incubated aerobically in a shaking water bath at 56 °C. Just before starting this incubation, and at intervals thereafter, samples were diluted into ice-cold YPD. Survival was then determined by plating on YPD plates and counting of the colonies formed over 3 days at 37 °C.

**RESULTS**

**Determining the temperature upshift that leads to optimal induction of the heat-shock response in H. polymorpha**

In S. cerevisiae, the heat-shock response is maximally activated by the largest upshifts of temperature, to a final temperature of around 39 °C (Kirk & Piper, 1991). The latter also approximates to highest temperature of growth for S. cerevisiae. In vivo protein pulse-labelling in H. polymorpha indicated maximal Hsp induction with temperature upshifts to 47–49 °C, conditions that induced massive synthesis of just a few Hsps (Fig. 1). The maximal temperature of growth of this strain on glucose media was 49-5 °C. Thus in H. polymorpha, as with S. cerevisiae, the
Fig. 1. Determination of the optimum temperature for induction of the *H. polymorpha* heat-shock response. Glucose-grown cells were labelled for 10 min, both in balanced 37 °C growth (second lane from left), or between 5 and 15 min following a temperature shift from 37 °C to the temperature indicated. Positions of molecular-mass markers are indicated to the left of the figure, and positions of major Hsps to the right.

optimal temperatures for Hsp induction approximate to the highest temperature of growth. Accordingly, 49 °C was used as the heat-shock temperature in most of our subsequent *H. polymorpha* experiments. Simultaneous with the Hsp synthesis that it induces is a suppression of the synthesis of most other proteins (Fig. 1). *H. polymorpha* that was growing on methanol as a carbon source, conditions that cause massive peroxisomal proliferation (Gellissen, 2000; Hollenberg & Gellissen, 1997; Sudbery, 1996; van Dijk *et al.*, 2000), displayed a heat-shock response essentially similar to that of cells growing on glucose (Fig. 2a). Pulse-labellings under conditions of temperature upshift, then downshift, revealed that the heat-shock response of glucose-grown *H. polymorpha* is rapidly switched off when cultures are downshifted from 49 °C to 30 °C (Fig. 2b).

The strongly heat-induced Hsps of *H. polymorpha* were denoted Hsp104, Hsp90, Hsp70 and Hsp26, since the first three exactly co-migrate on one-dimensional (1D) SDS gels with the equivalent Hsps of *S. cerevisiae* heat shocked to 39 °C (not shown). The *H. polymorpha* protein denoted Hsp26 (Figs 1, 2 and 3) actually migrates slightly slower than the *S. cerevisiae* Hsp26 on 1D gels, but is almost certainly the *H. polymorpha* equivalent of the latter protein.

**Heat shock induces transient Hsp synthesis and cell cycle arrest in *H. polymorpha***

The heat-shock response is transient in aerobic cultures of *H. polymorpha* suddenly subjected to a temperature upshift from 30 °C to 49 °C. Hsps are synthesized at an extremely high level during the first 15 min at the latter temperature, but this Hsp synthesis then declines with further 49 °C maintenance (Fig. 3a). There is no appreciable thermal death of *H. polymorpha* maintained at 49 °C (Cabeça-Silva & Madiera-Lopes, 1984) but we observed that these cells, initially in growth at 30 °C and then shifted to 49 °C, rapidly accumulated as unbudded cells at the latter temperature (not shown). This period of cell stasis lasted 4–6 h after the shift to 49 °C, during which time there was no increase in cell number. Thereafter, growth slowly resumed, eventually achieving the rate for balanced 49 °C growth. This prolonged stasis with heat shock appears to be a peculiar feature of the *H. polymorpha* heat-shock response. *S. cerevisiae* also shows a transient cell cycle arrest when abruptly transferred to its maximum growth temperature, accumulating transiently as unbudded cells (Rowley *et al.*, 1993). It appears, however, that *S. cerevisiae* cultures re-enter the cell cycle more rapidly when heat shocked to their maximum temperature of growth (achieving maximal proliferation rates for growth at 37–39 °C within 90 min of an upshift to these temperatures from 30 °C (Rowley *et al.*, 1993).

**Thermotolerance is induced both following a heat shock and during balanced growth at high temperature***

When *H. polymorpha* in mid-exponential growth at 30 °C was heated rapidly to 56 °C, rapid death ensued (viable cells decreasing by over two orders of magnitude within the first 10 min at the higher temperature; Fig. 4). A 15 min 30 °C to 49 °C heat shock prior to this shift to 56 °C provided protection against this thermal death (Fig. 4). Therefore,
brief 30°C to 49°C heat shock of *H. polymorpha* induces thermotolerance. Intriguingly, complete protection against the lethal temperature of 56°C was also apparent with cells that had been growing for several generations at 49°C (Fig. 4). *H. polymorpha* growing at its highest temperature of growth is therefore intrinsically thermotolerant.

Simultaneous imposition of anoxia at the time of a heat shock to 49°C induces sustained Hsp synthesis in *H. polymorpha*

*H. polymorpha* is an obligate aerobe, in contrast to *S. cerevisiae*, which can grow in the complete absence of oxygen on condition that it is provided with a source of sterols. The labellings in Figs 1, 2 and 3a were all conducted on actively respiring cultures of *H. polymorpha* maintained under normoxic conditions. In some of our preliminary pulse-labellings, small volumes of culture were transferred to capped microfuge tubes for the purposes of protein labelling. However, oxygen consumption by the actively respiring cells resulted in such cultures rapidly becoming anaerobic (as shown by the inclusion of a redox indicator, resazurin, in the medium). The gels of labelled proteins from these initial experiments (not shown) indicated a rather more sustained heat-shock response.

To determine if these early results might have been influenced by the oxygen deprivation, we repeated the experiment in Fig. 3a, but with the deliberate exclusion of oxygen at the time of temperature upshift (see Methods). Use of an oxygen electrode confirmed that hypoxic conditions had been rapidly established, as the heat-shocked respiring *H. polymorpha* consumed almost all of the available oxygen (to 10–12% atmospheric oxygen within 12–14 min, and to <2% over 30–40 min). There was, though, no reduction in viable cell count over the 165 min that these cells were maintained at 49°C. In these hypoxic heat-shocked cells, the induced Hsp synthesis was markedly sustained, remaining very high even after more than 2 h at 49°C (Fig. 3b). Indeed, Hsps were almost the only proteins that were being synthesized by such cells exposed to the combined stresses of a 49°C heat shock and hypoxia (Fig. 3b). A similar imposition of hypoxia to cultures in balanced 37°C growth did not lead to any strong Hsp induction (data not shown), showing that it is necessary to apply both stresses in order to obtain this sustained heat-shock response.

To determine whether hypoxia was generating this sustained heat-shock response in *H. polymorpha* by preventing the down-regulation of Hsp gene transcription that normally attenuates this stress response, we analysed the mRNA for the major heat-inducible Hsp70 of *H. polymorpha* (Titorenko *et al.*, 1996), both in normoxic and in hypoxic cultures heat shocked from 30°C to 49°C (Fig. 5). We were careful to use
conditions that exactly replicated those used for the protein pulse-labelling studies in Fig. 3. Northern blot analysis (Fig. 5a) revealed the Hsp70 mRNA undergoing an initial rapid increase, then decreasing in the normoxic culture maintained at 49 °C as the heat-shock response was down-regulated. In contrast, the levels of the Hsp70 mRNA were induced and then sustained in the culture that became hypoxic as it was maintained at 49 °C (Fig. 5a). Primer extension mapping of the 5′ termini on HphSA1 gene transcripts in the same 0, 15, 75 and 120 min RNA samples. DideoxyATP- (ddA) and dideoxyTTP- (ddT) containing DNA sequencing reactions, prepared using the same 5′-end labelled primer and pHSA1 plasmid as template, were run on the same gel alongside the primer extension reactions.

**DISCUSSION**

In this study, we investigated the heat-shock response of *H. polymorpha*, a species that grows to 49 °C and which, unlike *S. cerevisiae*, does not display any appreciable thermal death at its highest temperatures of growth (Cabeca-Silva & Madiera-Lopes, 1984; van Uden, 1984). The *H. polymorpha* heat-shock response displays many of the characteristics of the same response of *S. cerevisiae*, namely that it is induced at around the maximum temperature of growth (Fig. 1), is normally transient (Fig. 3a), and leads to a rapid induction of both trehalose and 56 °C thermotolerance (Fig. 4; also Reinders et al., 1999). An earlier study showed that the heat-induced synthesis of trehalose is not required for the high-temperature growth of *H. polymorpha*, but is essential for this yeast to survive at a higher, more extreme temperature (56.5 °C) (Reinders et al. 1999).

*H. polymorpha* is an obligate aerobe, such that it is not possible to deprive it totally of oxygen for extended periods. Nevertheless, the ability of hypoxia to prevent the normal attenuation of the heat-shock response (Figs 3, 5) indicates that oxygen, presumably respiratory chain activity, is required for the feedback regulation that normally renders the heat-shock response transient. A sustained heat-shock response is also seen in many systems upon loss of Hsp90 chaperone activity (Duina et al., 1998; Harris et al., 2001; Zou et al., 1998b). One Hsp90 mutation (corresponding to the E381K hsp82 allele) causes *S. cerevisiae* cells to display extremely high levels of Hsp synthesis, even at low temperatures of growth (Harris et al., 2001).

Heat-shock transcription factor (HSF) is the transcriptional regulator of the heat-shock response in eukaryotic cells. In *in vitro* studies using recombinant forms of HSF from *Drosophila* and yeast initially revealed that HSF may act as a direct sensor of physiological changes in temperature and oxidative state (Lee et al., 2000; Zhong et al., 1998). More recently, it was shown that recombinant mammalian HSF1 senses both heat and hydrogen peroxide directly through the reversible formation of two redox-sensitive disulphide bonds (Ahn & Thiele, 2003). This formation of disulphide-bonded HSF1 leads, in turn, to the HSF1 undergoing homotrimerization, nuclear import, and acquiring DNA-binding capability (Ahn & Thiele, 2003; Morimoto et al., 1997). While higher organisms generally have multiple forms of HSF (Morimoto et al., 1997), yeasts generally have just a single, essential HSF (Chen et al., 1993). The latter is regulated differently from mammalian HSF, in that it lacks redox-sensitive thiol groups (Ahn & Thiele, 2003) and exists constitutively homotrimerised in the yeast nucleus (Chen et al., 1993; McDaniel et al., 1989; Sorger, 1991). Recent work indicates that up to 3 % of *S. cerevisiae* genes may be subject to HSF regulation (Hahn et al., 2004).

The HSF of unstressed cells exists in association with chaperones, notably proteins of the Hsp70 family (Abravaya et al., 1992; Bonner et al., 2000; Shi et al., 1998). A widely held view is that high temperature causes a partial unfolding of intracellular proteins, and that these are then questered by Hsp70-family chaperones. This leaves HSF unchaperoned and therefore able to activate the heat-shock response. Subsequently, the response rapidly elevates levels of Hsp70, thereby allowing Hsp70 to rebind HSF, forcing this HSF to reform into the original inactive HSF–Hsp70 complex. There is considerable support for such a model. In *E. coli*, a variety of abnormal proteins are found to cause induction of the heat-shock response, provided that they are reasonably stable so that their levels can build up within the cell (Parsell & Sauer, 1989). In yeast, high levels of mistranslation (Grant et al., 1989; Trotter et al.,
2001), inhibition of the proteasome (Lee & Goldberg, 1998) and low levels of Hsp70-family proteins (Bonner et al., 2000; Craig & Jacobsen, 1984) can all activate the response. Similarly, the injection of abnormal proteins into Xenopus oocytes triggers a heat-shock response (Ananthan et al., 1996), while the human HSF is activated by treatments that induce the formation of glutathione–protein mixed disulphides (Zou et al., 1998a). Despite this wealth of evidence, a number of findings in microbes are inconsistent with the primary induction signal for the heat-shock response being unfolded protein. In the blue-green alga Synechocystis, there is a strong correlation between the degree of membrane order in thylakoids and the threshold temperature for activation of the heat-shock response (Vigh et al., 1998). In S. cerevisiae, the temperature optimum for the induction of the response is dramatically affected by membrane lipid content (Chatterjee et al., 1997). Reduced plasma membrane potential also suppresses the response (Panaretou & Piper, 1990). These findings are therefore evidence for a membrane sensor of heat stress.

Oxygen also appears to be important in the yeast heat-shock response. Superoxide is a strong inducer of S. cerevisiae HSF (Lee et al., 2000). Furthermore, anaerobic S. cerevisiae cultures, as well as both aerobic and anaerobic cultures of respiratory-deficient S. cerevisiae petites that lack an assembled respiratory chain, fail to show any HSF activation in response to heat shock (K. Hatzixanthis, M. Mollapour and P.W. Piper; unpublished observations). It appears therefore that an assembled functional mitochondrial respiratory chain may be a requirement for S. cerevisiae cells to mount a heat-shock response. This study provides yet further evidence for the importance of oxygen in the heat-shock response of yeasts, showing it to be required for the attenuation of this response in H. polymorpha (Figs 3 and 5).

The protein chaperones induced by the heat-shock response are thought to serve an important function in protection against protein damage (Ellis & van der Vries, 1991; Gething & Sambrook, 1992; Parsell & Lindquist, 1993; Welch, 1991). By binding to exposed hydrophobic surfaces, they help to prevent protein aggregation and, in certain cases, can even help to refold partially denatured proteins (Lindquist & Kim, 1996). The high Hsp synthesis induced by a heat shock of H. polymorpha to 49 °C (Figs 1, 2a and 3a) occurs simultaneously with growth arrest. Nevertheless, it declines rapidly (Fig. 3a), long before cell division is resumed. In an earlier study, the gene for heat-inducible Hsp70 analysed in Fig. 5 was both deleted and overexpressed in H. polymorpha (Titorenko et al., 1996). Remarkably, loss of this gene was found to increase survival, and its overexpression to decrease survival at 56 °C (Titorenko et al., 1996: a study that did not report the effects on growth at 49 °C). The available data therefore does not support the argument that elevated Hsp synthesis makes a major contribution to the high-temperature growth and survival of H. polymorpha.

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