

Mitochondrial Hsp60, Resistance to Oxidative Stress, and the Labile Iron Pool Are Closely Connected in *Saccharomyces cerevisiae**

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In the present study, we have analyzed the role of the molecular chaperone Hsp60 in protection of *Saccharomyces cerevisiae* against oxidative damage. We constructed mutant strains in which the levels of Hsp60 protein, compared with wild-type cells, were four times greater, and the addition of doxycycline gradually reduces them to 20% of wild-type. Under oxidative-stress conditions, the progressive decrease in Hsp60 levels in these mutants resulted in reduced cell viability and an increase in both cell peroxide species and protein carbonyl content. Protection of Fe/S-containing enzymes from oxidative inactivation was found to be dose-dependent with respect to Hsp60 levels. As these enzymes release their iron ions under oxidative-stress conditions, the intracellular labile iron pool, monitored with calcein, was higher in cells with reduced Hsp60 levels. Consistently, the iron chelator deferoxamine protected low Hsp60-expressing cells from both oxidant-induced death and protein oxidation. These results indicate that the role of Hsp60 in oxidative-stress defense is explained by protection of several Fe/S proteins, which prevent the release of iron ions and thereby avert further damage.

Heat shock proteins (Hsps)¹ are a family of proteins that act both under normal (where Hsps account for 5–10% of the total protein) and stress conditions (where they are overexpressed). Stress situations include heat shock, glucose deprivation, exposure to free radicals, infection by pathogens, and tissue injuries (1, 2). A common aspect of these stress-inducing conditions is that they give rise to proteins with non-native conformations. All known stresses, when sufficiently intense, induce Hsp expression. Hsps are therefore also referred to as stress proteins and constitute an important part of the stress response. Hsps are highly conserved among species and carry out such essential functions as protein translocation, folding,

and assembly under normal cellular conditions (3, 4). As a result, they have also been called “molecular chaperones.”

Many members of the HSP60 family are constitutively expressed and are found in abundance in prokaryotes, chloroplast, and mitochondria. Hsp60 is an essential mitochondrial chaperone and promotes the folding of many proteins imported into the mitochondrial matrix. It also directs several proteins to the intermembrane space (5–7). It is generally assumed that mitochondrial Hsp60 works according to a similar mechanism to its prokaryotic homologue, GroEL. GroEL is composed of 14 monomers arranged as a double ring, with a 7-mer in each. In its central cavity, substrate proteins can be transiently sequestered from the medium and allowed to mature in solitary confinement. The 10-kDa co-chaperonin (Hsp10 or GroES in bacteria) exists as a 7-mer ring-shaped oligomer. Newly imported proteins are released by an ATP-dependent reaction regulated by the co-chaperone Hsp10 (8–9). In short, unfolded proteins can be bound inside the central cavity of the large Hsp60. ATP binding and hydrolysis induces a large conformational shift, which changes the properties of the binding sites and opens the cavity. The substrate is eventually released in the folded conformation, possibly after several cycles of binding and release (10). In *Saccharomyces cerevisiae*, Hsp60 is essential for viability at all temperatures (this is consistent with its constitutive expression), and its concentration in the mitochondrial matrix is also increased 2- to 3-fold at 42 °C (6). Such induction reflects an increased requirement for this chaperonin to protect pre-existing proteins from denaturation and aggregation.

Davidson *et al.* (11, 12) demonstrated that in yeast, anaerobic cells are several orders of magnitude more resistant to heat shock than aerobic cells. Aerobic heat stress also led to an increase in mitochondrial membrane damage, which was reduced 100-fold under anaerobic conditions. The authors showed that heat stress generates oxidative stress, supporting the idea that antioxidant enzymes play a major role in protection against heat-induced cell death in yeast. Mutants deleted for the antioxidant genes catalase, superoxide dismutase, and cytochrome *c* peroxidase were more sensitive to the lethal effect of heat shock than isogenic wild-type cells. Overexpression of catalase and superoxide dismutase genes caused an increase in thermotolerance (11). A common signal has been proposed for heat stress and oxidative stress in cell culture, suggesting that exposure of the hydrophobic domains of proteins represents the most common early signal in the activation pathway of the heat shock factor (13).

Yeast can adapt to H₂O₂ and superoxide-generating drugs by inducing two distinct but overlapping stimulons (14, 15). The H₂O₂ stimulon includes the induction of many Hsps, with Hsp104 and Hsp12 being those most highly induced (14- and 10-fold, respectively) (16). Some other Hsps have been directly related with conferring resistance against oxidative stress (1). These include the cytoplasmic Hsp70, which is highly inducible

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¹ The abbreviations used are: Hsp, heat shock protein; DFO, deferoxamine; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; LIP, labile iron pool; YPD, yeast extract-peptone-dextrose medium; YPG, yeast extract-peptone-glycerol medium; ROS, reactive oxygen species.

(17), and the newly described chaperone Hsp33 which plays an important role in the bacterial system toward oxidative stress. To date, Hsp33 is the only chaperone whose function is regulated by environmental redox conditions, and it is directly activated by disulfide bonds formation (18). The non-ATP-dependent Hsps (such as Hsp33) have been referred to as "holdases," in contrast to the ATP-dependent "foldases" (such as Hsp60 and Hsp70) (19). Holdases bind stably to folding intermediates and suppress lethal aggregation, and they also interact with foldases, allowing the efficient release of intermediates and their successful refolding to their native state when conditions return to normal. However, with the exception of *Escherichia coli* Hsp33, which has been shown to protect oxidatively damaged proteins from irreversible aggregation (18), very little is known about the mechanisms by which Hsps protect against oxidative stress.

It is interesting to note that in previous work, we identified mitochondrial Hsp60 in yeast (20) and DnaK (the bacterial Hsp70) in *E. coli* (21) as major oxidized proteins under oxidative stress. Although members of different Hsp families, this parallelism led us to study their physiological relevance both in *E. coli* and *S. cerevisiae* upon oxidative stress. Our group has also recently published a report on the protective role of DnaK of alcohol dehydrogenase E from oxidative damage (22). In the present study, the expression of the *HSP60* gene under a *tet* promoter allowed us to demonstrate the importance of Hsp60 in protecting yeast cells against oxidative stress. We also propose a possible mechanism to explain this protection linking Hsp60, Fe/S cluster enzymes, mitochondrial iron homeostasis, and oxidative damage.

EXPERIMENTAL PROCEDURES

Chemicals—Hydrogen peroxide (30% solution), doxycycline, bathophenanthroline sulfonate, menadione sodium bisulfite, 2,4-dinitrophenylhydrazine, mesylate derivative of deferoxamine (DFO), and dihydrorhodamine 123 were purchased from Sigma. Calcein-acetoxymethyl ester was from Molecular Probes. Acrylamide/bisacrilamide solution was supplied by Bio-Rad. Polyvinylidene difluoride membranes were from Millipore. The chemiluminescent detection kit was provided by Tropix.

Yeast Strains and Plasmids—*S. cerevisiae* CML128 (*MATa*, *ura3-52*, *his4*, *leu2-3,112*, *trp1*) was used as wild-type strain (23). Overexpression of Hsp60 protein was obtained after integration of *Bst*EII-linearized pMM132 plasmid at the chromosomal *LEU2* locus of CML128 cells. pMM132 is an integrative plasmid containing *HSP60* under the doxycycline-regulated *tetO₇* promoter and was derived from pCM190 (24). The strain carrying the integrated pMM132 plasmid was labeled MML332 and also contained the wild-type *HSP60* allele at its original chromosomal locus. Addition of the antibiotic repressed expression of the *tet* construction without affecting the wild-type gene, resulting in wild-type levels of Hsp60 expression. A conditional mutant in which *HSP60* expression was exclusively doxycycline-regulated was further obtained from the MML332 strain by deletion of the wild-type *HSP60* allele using the *kanMX4* cassette (25). The resulting strain, labeled MML336, was able to grow on glycerol in the absence of doxycycline (*HSP60* expressed), but growth was finally arrested upon addition of the antibiotic (*HSP60* transcription immediately repressed), confirming the need for Hsp60.

Growth Conditions—Yeast cells were grown aerobically at 30 °C in YPD medium (1% yeast extract, 2% peptone, 2% glucose) or in YPG medium (1% yeast extract, 2% peptone, 3% glycerol). Cells from CML128, MML332, and MML336 strains, growing exponentially under either fermenting (YPD) or respiring conditions (YPG), were diluted to $A_{600} = 0.002$ and $A_{600} = 0.0009$, respectively, in order to obtain cells with different Hsp60 levels. Doxycycline was added to 0, 2, and 4 $\mu\text{g/ml}$ concentration, and growth was allowed for 12 h in YPD medium and 25 h in YPG medium, until approximately reaching to $A_{600} = 0.5$.

Stress Conditions and Viability—Cells obtained as indicated in the previous paragraph (about 10^7 cells per ml) were challenged with hydrogen peroxide or menadione. These were directly added to the growth media under the conditions indicated for each experiment. Untreated cultures were incubated in parallel over the same periods. When indicated, the iron chelator DFO was added at 10 mM concentration 1 h

prior to the stress. Viability was determined by colony counts on YPD plates after 3 days of incubation at 30 °C and was expressed as the percentage of the corresponding control cultures.

Preparation of Cell Extracts—Cell extracts were prepared as described (26). Protein concentration was determined by the Bradford method. For Western blot experiments with anti-DNP, protein carbonyl groups were derivatized with 2,4-dinitrophenylhydrazine as published (27) with minor variations. Crude extracts were clarified by treatment with 50% volume of chloroform, vortexed at maximum speed for 1 min, and centrifuged at $10,000 \times g$ for 10 min. The upper buffer solution was then recovered. Protein concentration was adjusted to 4 mg/ml, SDS was added to the samples to a final concentration of 6%, and they were subsequently heated at 100 °C for 3 min. One volume of 10 mM 2,4-dinitrophenylhydrazine in 10% trifluoroacetic acid was added to 1 volume of the sample at 25 °C. The reaction was run for 10 min and was stopped by the addition of 1 volume of 2 M Tris, 10% glycerol, and 15% 2-mercaptoethanol. Samples were ready for loading on SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Western Blot Analysis—SDS-PAGE was performed according to Laemmli (28). Monoclonal antibody anti-Hsp60 was purchased from Stress-Gen and used at 1:8,000 dilution. It was applied in conjunction with a rabbit anti-mouse conjugated with alkaline phosphatase (1:25,000 dilution, from Tropix) as a secondary antibody. Polyclonal anti-Aco1 acetonitase (1:2,000 dilution, a kind gift of R. Lill, Marburg University) and anti-succinate dehydrogenase (1:1,000 dilution, from B. Lemire, University of Alberta) antibodies were also employed. Rabbit anti-dehydroloipoamide succinyltransferase (E_2 subunit of α -ketoglutarate dehydrogenase) antibodies were obtained after injection of a KLH-conjugated peptide ($^{193}\text{KKEAAPKKEVTEPKK}^{208}$, from Fundacio Bosch i Gimpera, University of Barcelona) and applied at a dilution of 1:2,500. The anti-2, 4-dinitrophenol antibody (from DAKO) was used at a dilution of 1:4,000. A goat anti-rabbit antibody conjugated with alkaline phosphatase (1:25,000 dilution, from Tropix) was used as a secondary antibody.

Measurement of Intracellular Oxidation Levels—The oxidant-sensitive probe dihydrorhodamine 123 (Sigma D1054) was used to measure intracellular oxidation levels in yeast, as described in Madeo *et al.* (29). Dihydrorhodamine 123 is an uncharged, nonfluorescent product that passively diffuses across most cell membranes where it is oxidized to rhodamine 123, and locates in the mitochondria. Dihydrorhodamine was added to the cell culture at 2.5 $\mu\text{g/ml}$ from a 2.5 mg/ml stock solution in ethanol, and cells were incubated for 1 h. Hydrogen peroxide (8 mM) was added as indicated, and an additional incubation of 1 h was performed. Cells were washed, resuspended in phosphate-buffered saline (10 mM phosphate buffer, 150 mM NaCl, pH 7.0), and viewed through a fluorescein optical filter (excitation, 465–495 nm; emission, 505), using a Nikon fluorescence microscope. Images were taken using a Sony camera system. Fluorescence intensity was measured within 15–30 min, from cells treated as described above, using a Shimadzu RF 5000 spectrofluorimeter (excitation, 490 nm; emission, 530 nm). The emission values were normalized by number of cells.

Enzyme Activities—Cell extracts were obtained using glass beads, and enzymatic activities were assayed as described in the respective references: malate dehydrogenase (30), succinate dehydrogenase (31), and aconitase (32). Activities were expressed as $\text{nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$.

Determination of LIP and Total Iron—The intracellular labile iron pool (LIP) was measured using calcein, a fluorescent metal-sensitive probe (33). In cell-free systems, calcein binds to both iron (II) and iron (III) with stability constants of 10^{14} and 10^{24} M^{-1} , respectively, which results in quenching its fluorescence (34). After cellular uptake of calcein-acetoxymethyl ester, intracellular esterases convert the molecule into a nonpermeable acid-form of calcein. Because calcein is insensitive to calcium and magnesium ions up to 1 mM at physiological pH and because intracellular concentration of other calcein-binding molecules is very low, it is generally accepted that the observed decrease in calcein fluorescence is due to iron binding. Calcein-acetoxymethyl ester loading of *S. cerevisiae* cells was performed on exponential phase cell cultures untreated or treated in the presence of 8 mM for 1 h. Cells were washed twice with phosphate-buffered saline and resuspended in the same buffer at 10^7 cells/ml. Cells were loaded with calcein-acetoxymethyl ester at a final concentration of 5 μM (from a 10 mM stock solution in dimethyl sulfoxide). After a 2-h incubation at 30 °C, cells were washed twice to remove unincorporated dye, resuspended in phosphate-buffered saline, and viewed through a fluorescein optical filter (excitation, 465–495 nm; emission, 505 nm) using a Nikon fluorescence microscope. Images were taken as described previously. Fluorescence intensity was measured within 30 min, from cells treated as described above, using a Shimadzu RF 5000 spectrofluorimeter (excitation, 485 nm; emission, 525 nm). The emission value was normalized by the

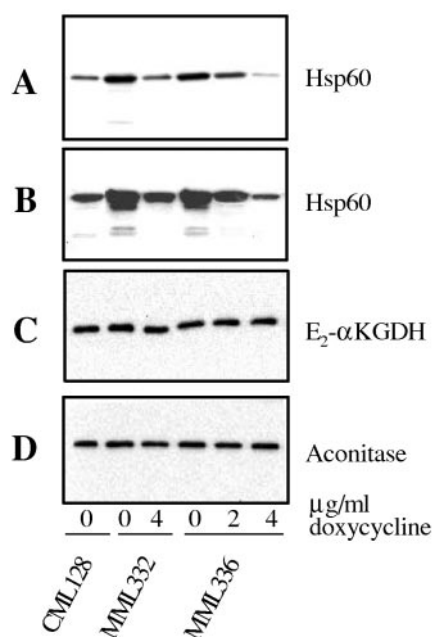


FIG. 1. Levels of Hsp60 can be regulated by doxycycline without affecting mitochondrial enzymes. Cells from CML128, MML332 and MML336 strains grown exponentially in YPD (A) or YPG medium (B–D) were treated with doxycycline, at time zero, at the indicated concentrations. After incubation for 12 h in the case of YPD and 25 h in the case of YPG, Western blot analysis of Hsp60 (A and B), E₂ subunit of α -ketoglutarate dehydrogenase complex (C) and aconitase (*Aco1*) (D) were performed.

number of cells. Total cellular iron was determined under reducing conditions (35), with bathophenanthroline sulfonate as chelator. In summary, 2×10^8 exponentially grown cells were washed twice in MilliQ water, resuspended in 500 μ l of 3% nitric acid, and digested for 12 h at 98 °C. After removal of the precipitated material by centrifugation, 400 μ l of supernatant was taken up and Fe³⁺ was reduced to Fe²⁺ with ascorbic acid (final concentration, 6 mg/ml). Ammonium acetate was added to obtain a final pH of 5.4. The difference between 535 nm and 680 nm absorbance was recorded before and after addition of bathophenanthroline sulfonate (final concentration, 34 μ g/ml). Cell numbers and mean cell volumes were determined using a Coulter Z2 counter (Beckman Coulter, Fullerton, CA). A blank sample containing all of the components except the cells was treated in parallel to measure nonspecific absorbance. Molar iron concentration was obtained from a standard curve prepared with ferrous ammonium sulfate.

RESULTS

Regulation of Hsp60 Levels by Doxycycline—Given the defective growth phenotype of *HSP60* null mutants, we constructed two conditional strain mutants in which the *HSP60* gene under the *tet* promoter was doxycycline regulable (see Experimental Procedures). It is possible to obtain cells with different amounts of Hsp60 protein from these strains, by adjusting time and doxycycline concentration. To quantify these levels, crude extracts were obtained from CML128 (wild-type), MML332, and MML336 cells grown in YPD or YPG with different doxycycline concentrations. YPD-grown (Fig. 1A) and YPG-grown (Fig. 1B) cells showed repression of *HSP60* gene expression by the antibiotic; similar results were recorded for both culture media. Control cells (no added doxycycline) displayed a 3.5- to 4-fold increase in Hsp60 expression in strain MML332 and a 2.5- to 3-fold increase in strain MML336 with respect to CML128 cells. Addition of 4 μ g/ml doxycycline to the MML332 strain reduced the amount of Hsp60 to that of the wild-type. When the same dose was added to the MML336 strain, Hsp60 levels were reduced to 15–20% of those found in the wild-type.

Neither Hsp60 overexpression nor underexpression affected growth in YPD at the indicated times and antibiotic concentra-

tions: all cells showed similar growth curves (data not shown). However, Hsp60 overexpression did slightly increase duplication time for cells grown in YPG. In this medium, cells from strain MML332 duplicated in 260 min, but in only 240 min when doxycycline was added at 4 μ g/ml (data not shown). Hsp60 underexpression, even at levels of around 20% did not affect viability in any media. All cells remained viable under these conditions (data not shown).

It is well known that the absence of Hsp60 causes imported proteins to fold incorrectly and to aggregate into insoluble complexes (36). We wanted to determine whether the mitochondria were still functioning properly under our conditions. Western blot detection of two enzymes reported to need Hsp60 for proper folding, α -ketoglutarate dehydrogenase (7) and aconitase (37), showed no differences (Fig. 1, C and D). This indicated that even at below normal levels, no insoluble aggregates were produced and no degradation of these proteins took place.

Strain MML336 arrested growth after about 18–22 h in YPD and 29–34 h in YPG, following the addition of 4 μ g/ml doxycycline. However, even at this point, cells were still able to almost completely recover growth once doxycycline was removed from the medium (data not shown).

Viability Increases after Oxidative Stress in Cells Overproducing Hsp60—The effect of Hsp60 in protection against heat stress was first described 10 years ago (36), but much less is known about its effects upon oxidative stress. We used our doxycycline-regulable strains growing in YPG as a model system to investigate how varying the quantity of this chaperonin may affect viability against menadione (a superoxide-generator compound) and H₂O₂. As shown in Table I, greater viability was obtained from cells with higher levels of Hsp60, and resistance was dose-dependent. Differences were especially clear following menadione treatment. As a control, adding the antibiotic to wild-type strain (CML128) at the maximum concentration used (4 μ g/ml) did not affect resistance to oxidative stress (data not shown).

During the stationary phase, a general stress response is required to reduce steady-state levels of damaged cell components that cannot be diluted out through cell division (38). To investigate whether increasing concentrations of Hsp60 have a protective effect on the stationary phase, cells from strains MML332 and MML336 were grown in YPD medium for 10 days and then treated with 50 mM H₂O₂ for 2 h. Our results showed that whereas in strain MML332 5% of the cells remained viable, in strain MML336 only 0.5% remained viable.

Hsp60 Plays a Protective Role against Protein Oxidative Damage—Carbonyl formation has been extensively used as a marker for protein oxidation (20, 21, 39, 40). We used this methodology to analyze the effect of challenging YPG-grown cells (from MML332 and MML336 strains treated with different concentrations of doxycycline) with 10 mM menadione or 8 mM H₂O₂ for 1 h. Western-blot anti-DNP revealed an increased level of protein damage in cells with lower Hsp60 levels (Fig. 2A). When menadione was used it was observed that two major proteins became targets for oxidative damage. As described in Cabiscol *et al.* (20) these were the mitochondrial enzymes dehydroliipoamide acetyltransferase (E₂ subunit of pyruvate dehydrogenase) and dehydroliipoamide succinyltransferase (E₂ subunit of α -ketoglutarate dehydrogenase). Hsp60 appeared as a major oxidized band under H₂O₂ stress. It is worth noting the strong oxidation of Hsp60 observed in the western pattern when its concentration declined (Fig 2A, strain MML336 with 2 μ g/ml doxycycline treated with H₂O₂). Similar results were obtained when cultures were grown in YPD medium (Fig. 2C). The presence of the antibiotic, added to wild-type cells

TABLE I
Effect of Hsp60 levels on survival under oxidative stress

Cells grown in YPG were treated with doxycycline at the indicated concentrations for 25 h and challenged with 50 mM menadione (60 min) or 10 mM H₂O₂ (120 min). DFO (10 mM) was added to the cultures 60 min before menadione or hydrogen peroxide. Cell viability is expressed as the percentage of the corresponding control cultures with an S.D. less than 15%.

Strain	Doxycycline ($\mu\text{g/ml}$)	Survival			
		Menadione	Menadione + DFO	H ₂ O ₂	H ₂ O ₂ + DFO
MML332	0	13	18	42	43
MML332	4	0.2	6	16	35
MML336	0	18	23	47	55
MML336	2	9	20	23	40
MML336	4	0.5	7	10	23

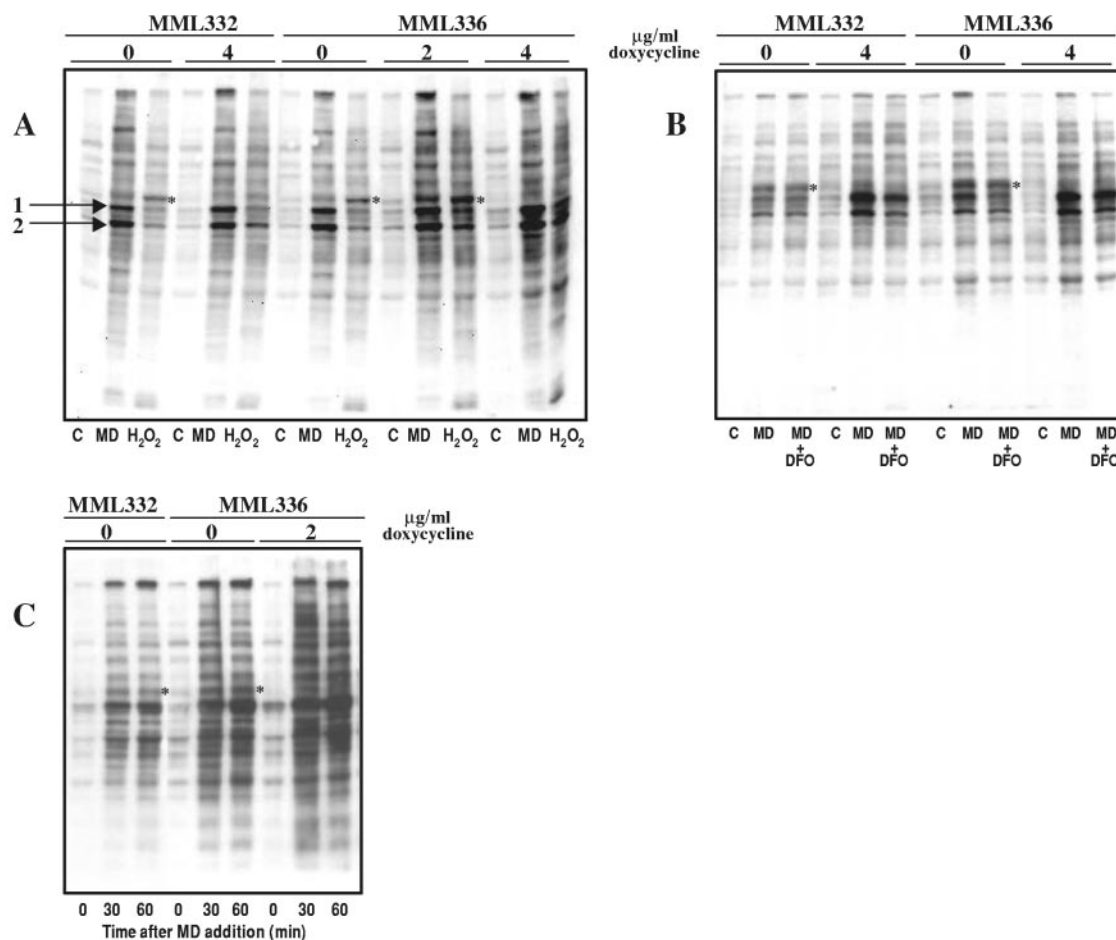


FIG. 2. Hsp60 protects proteins from oxidation upon oxidative stress. Cells from MML332 and MML336 strains were grown to exponential phase in YPG (A and B) or YPD medium (C) as described in Fig. 1, with the indicated doxycycline concentrations. A, cells were treated with 10 mM menadione (MD) or 8 mM H₂O₂ for 1 h. B, when indicated, cells were treated for 2 h with 10 mM DFO prior to menadione stress (10 mM, 1 h). C, cells were treated with 20 mM MD for 30 and 60 min. In all cases, untreated cells were used as a control. Oxidatively damaged proteins were detected by anti-DNP Western blot. Arrows indicate E2 subunits of both pyruvate dehydrogenase (1) and α -ketoglutarate dehydrogenase (2), which were severely oxidized under MD treatment. Asterisks indicate Hsp60, which is also seen as a carbonylated protein.

(CML128 strain), at the maximum concentration used, did not modify the oxidative pattern of damaged proteins under oxidative stress (data not shown).

Peroxide Levels Increase in Hsp60 Depleted Cells under Oxidative Stress—To further investigate the role of Hsp60 under oxidative stress situations, we tested levels of reactive oxygen species (ROS) by incubating the cells with dihydrorhodamine 123. This fluorescent probe has been extensively used to investigate oxidative bursts, as it reacts with H₂O₂ and other reactive oxygen intermediates (29, 41, 42). This substance accumulates in the cell where it is oxidized to the fluorescent chromophore rhodamine by ROS. In our model, doxycycline was added to MML332 and MML336 cell cultures grown in YPG, as described under “Experimental Procedures.” Dihy-

drorhodamine 123 was added at a 2.5 $\mu\text{g/ml}$ final concentration, and cells were incubated for 1 h. At that point, the cells were challenged with 8 mM H₂O₂ for 1 h, washed, and resuspended in PBS. Cells were immediately viewed under a fluorescence microscope (Fig. 3A), and fluorescence intensity was measured as described under “Experimental Procedures” (Fig. 3B). Strain MML336 treated with 4 $\mu\text{g/ml}$ of the antibiotic (Hsp60 levels were 15–20% of the wild-type) showed a very intense rhodamine fluorescence corresponding to a 7-fold increase with respect to unstressed cells. Cells from MML336 strain without doxycycline showed a faint fluorescence under the microscope (about 2-fold increase), and those treated with 2 $\mu\text{g/ml}$ doxycycline presented an intermediate fluorescence level (about 5-fold increase). Fluorescence in cells from the MML332

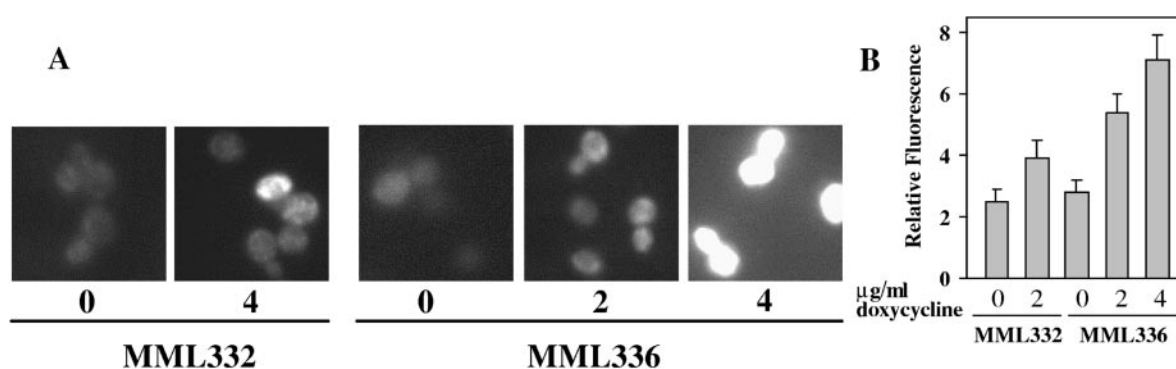


FIG. 3. Under oxidative stress, Hsp60 depletion increases peroxide levels. Cells from MML332 and MML336 were grown in YPG medium and treated with doxycycline as described in Fig. 1. Dihydrorhodamine 123 was added to the cells and 1 h later, challenged with 8 mM H_2O_2 for 1 h. A, fluorescence intensity visualized in a fluorescence microscope. B, relative fluorescence (represented as induction fold) with respect to control (unstressed) conditions. Each value represents the mean \pm S.D. from three separate experiments.

strain without antibiotic was also low with only a 2-fold increase. When this strain was treated with 4 μ g/ml doxycycline, fluorescence was similar to that seen in strain MML336 plus 2 μ g/ml doxycycline, which was consistent with their similar levels of Hsp60. In summary, from this experiment we can conclude that upon oxidative stress, Hsp60 deficient cells presented an increased ROS levels that could account for the majority of the phenotypes previously described.

Hsp60 Protects Mitochondrial Enzymes from Oxidative Inactivation—To investigate a possible mechanism to explain the results obtained with the fluorescent probe, the activities of aconitase and succinate dehydrogenase, two enzymes containing Fe/S clusters, were tested. These types of proteins are especially important because these clusters are very sensitive to oxidation (43–46) and liberate iron ions (47, 48). This free iron would enhance further ROS production throughout the Fenton and Haber-Weiss reactions (49).

To test the effect of Hsp60 levels on the oxidative inactivation of these enzymes, cells from MML332 and MML336 strains, grown in YPG, were treated with doxycycline as described under “Experimental Procedures.” Enzyme activities were measured before and after treatment with 8 mM H_2O_2 for 1 h (Fig. 4A). It is interesting to note that without additional stress (control cells), both aconitase and succinate dehydrogenase activities were dose-dependent with respect to Hsp60 levels. Furthermore, after addition of H_2O_2 to the cultures, the percentage of enzyme inactivation was greater in cells presenting lower amounts of Hsp60 with respect to their untreated controls. Western blot analysis before and after the addition of H_2O_2 showed apparently no effect on aconitase or succinate dehydrogenase levels (Fig. 4B). Again, no antibiotic effect was observed when added to wild-type cells (data not shown). Inhibition of Fe/S enzymes could give a clue to the nature of the phenotype observed upon oxidative challenge.

As a control, malate dehydrogenase, an enzyme with no Fe/S cluster, which has been described as being resistant to oxidative stress (45, 46), showed no significant differences between control samples and slightly increased activity after H_2O_2 treatment (Fig. 4A).

Intracellular LIP Increases When Hsp60 Decreases—Inactivation of Fe/S enzymes such as aconitase and succinate dehydrogenase (as described above) could result in the release of their iron ions. Calcein, a fluorescent metal-sensitive probe, was used to test this hypothesis. Calcein fluorescence is quenched when it binds the intracellular LIP.

Using our model, cells from MML332 and MML336 strains grown in YPG for 25 h with different doxycycline concentrations, were untreated or treated in the presence of 8 mM H_2O_2

for 1 h. Cells were loaded with calcein-acetoxymethyl ester (see “Experimental Procedures”) for 2 h, viewed under a fluorescence microscope (Fig. 5A) and fluorescence intensity was measured as described under “Experimental Procedures” (Fig. 5B). From these results it was clear that (i) H_2O_2 stress increased LIP, as there was a decrease in fluorescence compared with control conditions; (ii) In each mutant, the decrease in fluorescence after the stress was dependent on Hsp60 content; and (iii) even under control conditions (no external stress), a strong correlation existed between fluorescence and Hsp60 levels. Total iron was measured using bathophenanthroline sulfonate as described under “Experimental Procedures” to rule out the possibility of iron accumulation inside the cell could be different from one strain to another. Results demonstrated that there were no significant differences in total cell iron ($78 \pm 15 \mu$ M) between strains CML128, MML332, and MML336, regardless of the presence or absence of doxycycline.

Deferoxamine Protects Low Hsp60-expressing Cells—In this system, iron release seems an important factor in oxidative stress sensitivity. Consequently, a ferric iron chelator capable of permeating the cell, like DFO (50), should be able to rescue cells from oxidant-induced death, as it has been observed in fibroblasts from patients affected of Friedreich’s ataxia (51). As shown in Table I, protection by DFO was especially relevant in cells presenting low levels of Hsp60; in Hsp60-overexpressing cells, the effect was negligible. As a control, treatment with 10 mM DFO for 1 h had no effect on cell viability (data not shown). The same approach was used to test the effect of the iron chelator on protein carbonylation (Fig. 2B). Addition of DFO to cells displaying low Hsp60 levels, prior to menadione stress, resulted in a clear decrease in protein oxidation when compared with the same stressing conditions in the absence of DFO. Fig. 2B also showed that, regardless the presence of DFO, cells with high levels of Hsp60 (MML332 without doxycycline) presented the same level of protein oxidation.

DISCUSSION

The mitochondrial electron transport chain consumes >90% of cellular oxygen, and superoxide anions are generated as minor by-products. Despite the high level of antioxidant systems found in this organelle (52), mitochondrial damage is probably the most important after oxidative stress (43, 53). We previously described that when yeast cells are challenged with oxidative stress, several mitochondrial proteins become selectively oxidized (20). These include, apart from Hsp60, α -ketoglutarate dehydrogenase and pyruvate dehydrogenase. Aconitase, although much less carbonylated, almost completely loses its activity. In addition, yeast null mutants of *sod1* (encoding

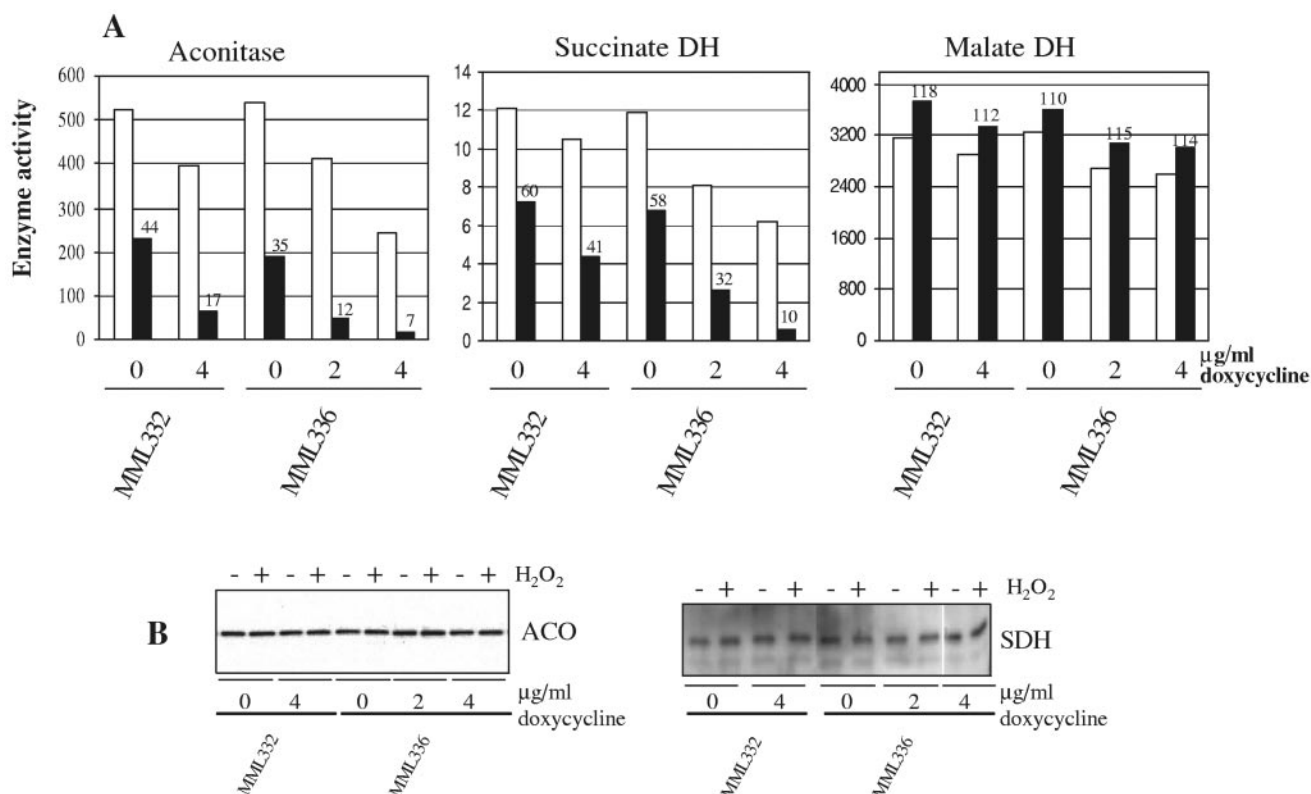


FIG. 4. Lack of Hsp60 negatively affects the activity of Fe/S enzymes upon oxidative stress. MML332 and MML336 cells growing exponentially in YPG medium were treated with doxycycline as described in Fig. 1. Cells were challenged with 8 mM H₂O₂ for 1 h. Unstressed cells were used as controls. *A*, enzyme activities (nmol·min⁻¹·mg⁻¹) in cell extracts were determined in control (*open bars*) and treated (*closed bars*) cells. The numbers on top of the *closed bars* indicate the percentage of remaining activity with respect to their untreated controls (set as 100%). Values given are means of at least three independent experiments with an S.D. of less than 5%. *B*, cell extracts were analyzed by Western blot using anti-aconitase (*Aco1*) and anti-succinate dehydrogenase (*Sdh2*) antibodies.

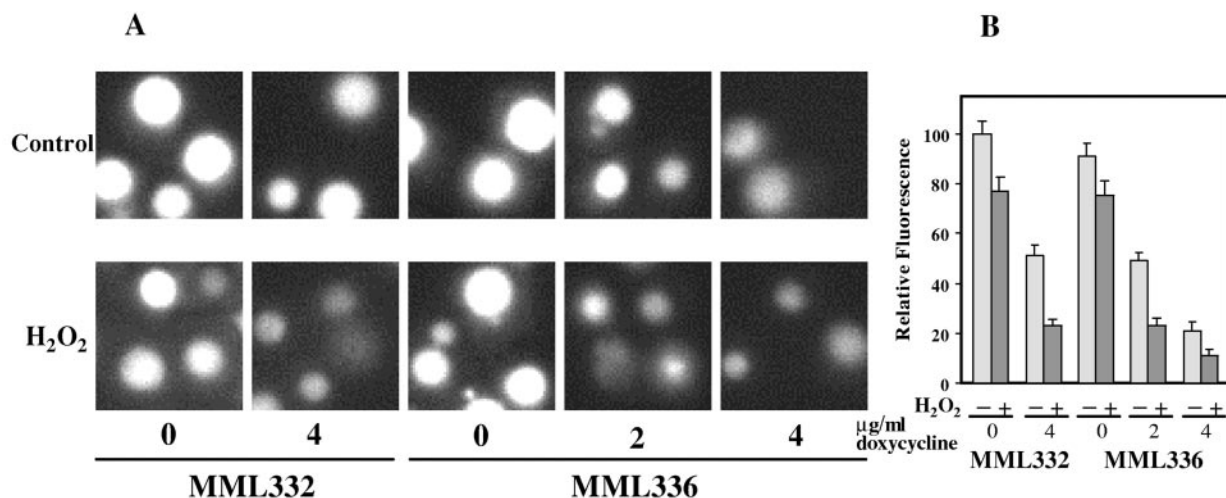


FIG. 5. Hsp60 protects cells from oxidative stress by reducing LIP. MML332 and MML336 cells grown to exponential phase in YPG medium were treated with doxycycline as described in Fig. 1. Cells were challenged with 8 mM H₂O₂ for 1 h. Unstressed cells were used as controls. Calcein-acetoxymethyl ester was added to the cells at 5 μg/ml for 2 h. *A*, fluorescence intensity visualized in a fluorescence microscope. *B*, relative fluorescence produced in cells untreated (*dotted bars*) and treated with H₂O₂ (*shaded bars*). Each value represents the mean ± S.D. from three separate experiments.

for Cu-Zn superoxide dismutase) present elevated protein carbonyls in mitochondria (54) and are respiration-deficient cells. Inhibition of respiration reverses their negative effect on long-term viability, where mitochondria exhibit a burst of ROS production (55). It is interesting to note that a fraction of SOD1, classically defined as cytosolic, has recently been reported to locate at the intermembrane space of mitochondria together with its copper chaperone (54).

It has been demonstrated that protein folding inside mitochondria are promoted through a successive action of the Hsp70 complex and Hsp60 complex (56). Yeast mitochondria contain three Hsp70 chaperone systems: (i) Ssc1, the main mitochondrial Hsp70, an essential protein that mediates the import of proteins into mitochondria from the cytosol and their subsequent protein folding, assembly, and degradation (57); (ii) Ecm10, a closely related protein, with an unclear role (58); and

(iii) Ssq1, reported to be 2,000 times less abundant than Ssc1 and implicated in the synthesis and assembly of Fe/S clusters (59). Hsp60 interacts, in an ATP-dependent manner, with other proteins and, in doing so, minimizes the probability of these other proteins interacting inappropriately with one another. It has been suggested that the majority of Hsps recognize hydrophobic regions exposed on the surface of partially misfolded proteins and facilitate refolding, thus preventing irreversible aggregation (36, 60). The importance of Hsp60 lies in both its abundance and, more importantly, its mitochondrial location.

To date, no report has been published explaining how Hsp60 protects cells from injuries associated to oxidative stress. In this report we have addressed the question using mutant strains in which the *HSP60* gene was under a *tet* promoter. These constructions allowed us to obtain cells containing various amounts of Hsp60. In this model, cells displaying higher doses of Hsp60 protein were more resistant to both H_2O_2 and superoxide anions, in terms of cell viability. Accordingly, protein carbonylation tested by Western blot showed that under oxidative stress, modifying Hsp60 levels affected the degree of protein damage although the targets were the same, as shown by the Western blot patterns. Also, an increase in intracellular ROS levels was observed—after oxidative stress—in cells presenting lower levels of Hsp60.

The role of Hsp60 under oxidative-stress conditions can be inferred from the observation that overexpression of this chaperonin protects two enzymes possessing Fe/S clusters from oxidative inactivation. Given the broad spectrum of substrates, we believe that Hsp60 may also protect any susceptible protein exposing hydrophobic regions. However, Fe/S enzymes are especially interesting because it is known that they liberate their iron after oxidation (48), producing additional stress (49). The presence of iron chelators has been shown to protect several types of cells from oxidative stress (21, 51, 61, 62). Metal ion toxicity is highlighted by the fact that null mutants of metallothioneins are very sensitive to oxidative stress (63).

In our model, total iron was not increased in cells underexpressing Hsp60. The “free” (*i.e.* non-protein-bound) iron referred to as LIP represents a part of the intracellular iron that is chelatable by commonly used chelators. LIP-associated iron exists in dynamic equilibrium in the cell with other sequestered iron forms and is bound to low affinity ligands that have yet to be completely identified, although substances such as ADP, ATP, GTP, and citrate are physiologically relevant (64). Because tissue homogenization may alter the existing equilibrium between free and bound iron, as well as its oxidation state (62), it was only possible to measure relative changes of LIP in intact cells after the advent of the use of calcein. In our hands, this iron-sensitive fluorescent probe showed that the ratio between ferrous and ferric ion, and/or the level of “free” iron was indeed altered. The results clearly demonstrated that cells overexpressing Hsp60 displayed reduced LIP after oxidative stress. Even more importantly, without additional stress, Hsp60 levels inversely correlated to LIP. The relevance of iron release was stated by using an iron chelator capable to permeate the cell. Addition of DFO to cultures prior to stressing compounds clearly preserved cell viability and decreased protein carbonyl formation significantly. These effects were observed in cells displaying Hsp60 levels equal to or below those of the wild-type cells. In cells with higher doses of the chaperonin, addition of DFO did not offer any additional advantage.

From these results, it was possible to propose a role for Hsp60 in protection against both endogenous and exogenous oxidative stress. Under oxidative stress, iron-dependent enzymes such as those presenting Fe/S clusters (*i.e.* aconitase and succinate dehydrogenase) and heme groups (*i.e.* cytochromes)

tend to release their metal ions to the medium. Free iron acts as a catalyst in several oxygen radical reactions (Fenton and Haber-Weiss reactions) generating toxic side products, such as the highly reactive hydroxyl radical (65). Partial unfolding, resulting from oxidative damage, may trigger the binding of Hsp60 to these proteins. Given that under an acute oxidative stress ATP levels drastically decrease (20, 46, 66), the capacity of Hsp60 to refold unfolded proteins will also be reduced. It has been suggested that upon stress, GroEL reduces its protein-folding activity and starts to act as a store for proteins, reverting to its normal function when conditions return to normal (67). Consistently, Hsp60 may act as a shield and prevent iron release, further protein damage and aggregation. It can also be deduced that cells with decreased Hsp60 levels will have a reduced capacity to protect protein from further oxidation that, in turn, will enhance a rise in LIP and the generation of ROS, promoting a deleterious cycle.

The role of molecular chaperones other than Hsp60 preserving the integrity of Fe/S clusters is also crucial to the cell; nevertheless, the mechanisms to perform it could follow different pathways. Mutations on *ssq1* and *jac1* genes, which are involved in synthesis and assembly of these clusters, alters iron homeostasis, leading to mitochondrial damage and inability to carry out respiratory metabolism (68, 69). In addition, yeast mitochondrial Hsp70s, Ssq1 and Ssc1, participate in the maturation of Yfh1 (70), a protein involved in iron homeostasis, which is the orthologue of the human protein frataxin. Mutations in the frataxin gene are associated with the neurodegenerative disease, Friedrich's ataxia.

It would be interesting to understand the role of free iron in several human diseases associated with defects in mitochondrial Hsp60. Systemic mitochondrial encephalomyopathy with multiple mitochondrial enzymes deficiencies has been attributed to the reduced synthesis of Hsp60 (71, 72). In congenital lactic acidemia, which is characterized by reduced activities of a number of mitochondrial enzymes, reduced levels of Hsp60 are suggested to be a more common cause of mitochondrial disease (73). More recently, a mutation in the gene encoding mitochondrial Hsp60 was reported as the primary cause of hereditary spastic paraplegia (74). Furthermore, Hsp60 overexpression (75) or induction (76) in rat neonatal cardiac cells protected them against simulated ischemia and reoxygenation-induced cell death. It did this by maintaining mitochondrial integrity and the capacity for ATP generation, both of which are crucial factors in determining survival (75). This protective property may be used in many therapeutic applications such as interventions associated with cardiovascular and retinal injuries, as well as in organ preservation and transplantation. However, because the role of Hsp60 in cardiovascular pathophysiology has already carefully studied (77), the associated risk needs to be evaluated.

The precise mechanism that explains why yeast Hsp60 and *E. coli* DnaK became carbonylated after oxidative stress remains open to discussion. However, several possibilities could be suggested. (i) Oxidation of these molecular chaperones may be a consequence of them acting as “shields” for proteins like aconitase and succinate dehydrogenase. It has been suggested that oxidized proteins may contain reactive species that may damage other proteins (78). (ii) A second possibility relates to their ATPase activity. ATP (and ADP) easily binds to divalent metal ions (79) such as iron and copper, which can perform metal-catalyzed oxidation after H_2O_2 treatment. (iii) The third possibility arises from an interesting report which, somewhat unexpectedly, reveals that Hsp60 and α -ketoglutarate dehydrogenase bind to mitochondrial DNA (80). These associated proteins would be damaged because iron ions bound to the

DNA backbone (81, 82) will trigger the formation of hydroxyl radicals by the Fenton reaction.

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METABOLISM AND BIOENERGETICS:
**Mitochondrial Hsp60, Resistance to
Oxidative Stress, and the Labile Iron Pool
Are Closely Connected in *Saccharomyces
cerevisiae***

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