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Oxidative Stress Promotes Specific Protein Damage in *Saccharomyces cerevisiae**

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We have analyzed the proteins that are oxidatively damaged when *Saccharomyces cerevisiae* cells are exposed to stressing conditions. Carbonyl groups generated by hydrogen peroxide or menadione on proteins of aerobically respiring cells were detected by Western blotting, purified, and identified. Mitochondrial proteins such as E2 subunits of both pyruvate dehydrogenase and α-ketoglutarate dehydrogenase, aconitase, heat-shock protein 60, and the cytosolic fatty acid synthase (α subunit) and glyceraldehyde-3-phosphate dehydrogenase were the major targets. In addition we also report the *in vivo* modification of lipoamide present in the above-mentioned E2 subunits under the stressing conditions tested and that this also occurs with the homologous enzymes present in *Escherichia coli* cells that were used for comparative analysis. Under fermentative conditions, the main protein targets in *S. cerevisiae* cells treated with hydrogen peroxide or menadione were pyruvate decarboxylase, enolase, fatty acid synthase, and glyceraldehyde-3-phosphate dehydrogenase. Under the stress conditions tested, fermenting cells exhibit a lower viability than aerobically respiring cells and, consistently, increased peroxide generation as well as higher content of protein carbonyls and lipid peroxides. Our results strongly suggest that the oxidative stress in prokaryotic and eukaryotic cells shares common features.

Cells growing in an aerobic environment need to cope with prooxidant conditions. Superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$) are normal byproducts of aerobic respiration (1). These reactive oxygen species (ROS)[1] also derive from external environmental factors such as redox active drugs, radiation, and heavy metals (1). As a result, ROS cause damage to proteins, lipids, and nucleic acids and thereby compromise cell viability. A common property of prokaryotic and eukaryotic cells is their ability to develop defenses against ROS. In *Escherichia coli*, the adaptive response to oxidative stress has been well characterized. OxyR and SoxR/SoxS are transcriptional regulators that control the expression of several proteins under H_2O_2 or $O_2^{\cdot-}$ stress, respectively (2–4). In addition, the *rpo*S-encoded σ^s subunit of RNA polymerase is involved in controlling the expression of several antioxidant defense genes. Our previous observations *in vivo* (5) indicated that in *E. coli* selective protein oxidative damage occurs when the cells were exposed to H_2O_2 or $O_2^{\cdot-}$ stress. Proteins such as alcohol dehydrogenase, elongation factor G, the heat-shock protein DNA K, and the β-subunit of F_0F_1-ATPase were identified as major targets. Those results gave a better comprehension of how such stressing conditions affects specific cellular processes.

In the yeast *Saccharomyces cerevisiae*, this adaptive response involves several transcription factors (Yap1, Yap2, Ace1, Mac1, and Hap1) that trigger the response to H_2O_2, $O_2^{\cdot-}$, and metal ions. The STRE element is present in the promoter of some of these inducible genes (6). The STRE-dependent induction of such genes also requires the binding of two zinc-finger proteins, Msn2 and Msn4. (7–9). Variations in the amounts of proteins, which are induced or repressed in yeast adaptive responses to hydrogen peroxide stress, have been described (10, 11) and analyzed (12).

Despite adaptive responses, cells exhibit a background level of oxidative damage to their macromolecules, especially those from mitochondria. About 2% of the oxygen consumed by the mitochondrial respiratory chain generate $O_2^{\cdot-}$ (13). There is a subsequent dismutation of $O_2^{\cdot-}$ to H_2O_2, and this molecule, in combination with Fe^{2+}, produces the highly reactive hydroxyl radical (14, 15). Increased production of these ROS is deleterious to mitochondria and thus to the metabolic and structural integrity of the cell.

Oxidative damage to proteins can be evaluated by the titration of carbonyl groups generated in some amino acid side chains during stress conditions (16, 17). The development of such methodologies has allowed the establishment of a correlation between protein oxidation and aging (18) and also with several diseases such as amyotrophic lateral sclerosis, Alzheimer's disease, respiratory distress syndrome, muscular dystrophy, Werner's syndrome, and progeria (19). Given that identification of proteins affected by oxidative stress *in vivo* has only been done in prokaryotic cells (5), we have used *S. cerevisiae* as a general model for eukaryotic cell type to: (i) identify oxidatively damaged proteins under different oxidative stress conditions; (ii) compare its oxidation targets with those of *E. coli* and evaluate the type of damage suffered by them when similar targets are shared; and (iii) test the physiological effects of the loss of function of some of these proteins.

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[1] The abbreviations used are: ROS, reactive oxygen species; H_2DCFDA, 2′,7′-dichlorofluorescein diacetate; YPD, yeast extract-peptone-dextrose medium; YPG, yeast extract-peptone-glycerol medium; α-KGDH, α-ketoglutarate dehydrogenase; PDH, pyruvate dehydrogenase; TBARS, thiobarbituric acid-reactive substances; E1, thiamine pyrophosphate-dependent 2-oxo-acid dehydrogenase; E2, dihydrolipoamide acyltransferase; E3, FAD-containing dihydrolipoamide dehydrogenase; DNPH, 2,4-dinitrophenylhydrazine.

EXPERIMENTAL PROCEDURES

Materials—Hydrogen peroxide (30% solution), menadione sodium bisulfite, 2,4-dinitrophenylhydrazine (DNPH), and protease from *Staphylococcus aureus* V8 were purchased from Sigma. Acrylamide/bisacrylamide solution was supplied by Bio-Rad. Polyvinylidene difluoride membranes (Immobilon Pseq) were from Millipore Corp. The chemiluminescent detection kit (Western Light) was from Tropix. 2′,7′-Dichorofluorescein diacetate (H_2DCFDA) was from Molecular Probes (Ref. D-399). Polyclonal rabbit antibodies against lipoic acid were donated by Luke Szweda (Case Western University).

Organisms and Growth Conditions—The strain used in this work was *S. cerevisiae* CML 128 (20). Yeast cells were grown exponentially at 30 °C in YPD medium (1% yeast extract, 2% peptone, 2% glucose) or YPG medium (1% yeast extract, 2% peptone, 3% glycerol) by incubation in a rotary shaker at 200 rpm. To calculate cell viability, appropriate dilutions of the cultures were spread on plates with solid YPD medium; these were incubated at 30 °C and colony-forming units were determined after 3 days. The level of cell viability in stressed cultures was determined relative to untreated control cultures taken at the same optical density. Cultures of *E. coli* K12 (kindly provided by E. C. C. Lin) were grown at 37 °C in minimal mineral medium (34 mM NaH_2PO_4, 64 mM K_2HPO_4, 20 mM $(NH4)_2SO_4$, 1 μM $FeSO_4$, 0.1 mM $MgSO_4$, and 10 μM $CaCl_2$) plus 0.2% glucose and aerated in a rotary shaker at 200 rpm.

Stress Conditions—Exponentially growing cells at 1×10^7 cells/ml were treated with hydrogen peroxide or menadione under the conditions indicated in each experiment. In preadaptation experiments, cells growing either in YPG or YPD were treated with a low dose of hydrogen peroxide (0.25 mM) for 30 min followed by 5 mM hydrogen peroxide for 45 min. The same time protocol was used for menadione using 1 and 40 mM, respectively. When desired 1% succinate was added to cultures. Cultures of *E. coli* were challenged with 10 mM menadione for the indicated period of time.

Preparation of Cell Extracts—Samples of *S. cerevisiae* for analytical Western blot experiments were taken from exponentially growing cultures (A_{600} = 0.6, 1×10^7 cells/ml) and prepared as described (21). For preparative purposes, cells from 1-liter cultures were broken with a French press (SLM Aminco) at a gauge pressure of 2200 p.s.i., using a FA-030 chamber. To obtain *E. coli* extracts, cells were disrupted with a French press at a gauge pressure of 900 p.s.i., using an FA-003 chamber. Crude extracts were clarified by centrifugation at 5000 rpm for 10 min. For Western blot experiments, samples were prepared as described previously (5). Protein concentration was determined by the Bio-Rad protein assay.

Quantification of Protein Carbonyls—Protein carbonyl content of crude extracts was measured according to the dinitrophenylhydrazine derivatization method described by Levine *et al.* (16). Quantification was performed using a Zorbax GF 250 high pressure liquid chromatography gel filtration column at 1 ml/min flow rate and kept at 25 °C. Absorbance at 276 and 370 nm was monitored using a Waters 996 diode array detector. Values of carbonyl content for crude extracts are given in nmol/mg protein.

Western Blot Analysis—Immunodetection of protein-bound 2,4-dinitrophenylhydrazones in crude extracts of *S. cerevisiae* and *E. coli* was conducted as described in Shacter *et al*. (17). The anti-2,4-dinitrophenol antibody (DAKO Ref. V0401) was used at a 1/4000 dilution. To detect lipoic acid bound to proteins, the antibody against lipoic acid was used at a 1/50,000 dilution. In both cases, the secondary antibody (goat anti-rabbit conjugated with alkaline phosphatase, Tropix) was used at a 1/25,000 dilution.

Isolation and Identification of Oxidatively Damaged Proteins—Two approaches were used to isolate the proteins of interest, ion exchange chromatography and preparative electrophoresis. Crude extracts (typically 250 mg of protein) were introduced on a Waters DEAE 15HR column using a fast protein liquid chromatography system. After a 20-min wash with solvent A (50 mM Tris-HCl, pH 7.5), a linear gradient from 0 to 50% of solvent B (50 mM Tris-HCl, pH 7.5, plus 0.5 M NaCl) was developed over 40 min at a flow rate of 5 ml/min. Collected fractions were derivatized with DNPH and analyzed by Western blot as described above. Preparative electrophoresis, sample preparation for NH_2-terminal sequencing, and limited proteolysis, used to further assess protein identification, were performed according to Tamarit *et al*. (5).

Enzyme Activities—To detect cytosolic enzyme activities cell extracts were broken using glass beads and assayed as described in the respective references: enolase (24), fatty acid synthase (25), pyruvate decarboxylase (26), and glyceraldehyde-3-phosphate dehydrogenase (27). To measure mitochondrial enzyme activities, yeast mitochondria were partially purified as described (28). α-Ketoglutarate dehydrogenase (α-KGDH) and pyruvate dehydrogenase (PDH) activities were measured as previously reported (29). Aconitase activity was determined by the cis-aconitate detection method (30).

TABLE I

Carbonyl content of crude extracts and cell viability after exposure to hydrogen peroxide stress

Cell viability is the percentage of the corresponding YPG and YPD cultures and the values are the mean of three independent experiments with a variation of ±5%. Carbonyl content, given in nmol of carbonyl/mg of protein, was determined after 45 min of treatment of the indicated H_2O_2 concentration. The values summarized here are mean values for three separate experiments with a variation of ±0.05. TBARS values are given in nmols/mg protein with a variation of ±0.01.

Culture conditions	Survival	Carbonyl content	TBARS
mM			
YPD control	100	0.51	0.040
0.25	72	0.78	
2	40	1.11	0.130
5	18	1.58	
5 (pretreated with 0.25 mM)	68	0.85	
YPG control	100	0.80	0.045
2	51	0.98	0.170
5	48	1.12	

Measurement of Intracellular Oxidation Level—The oxidant-sensitive probe H_2DCFDA was used to measure the intracellular oxidation level in yeast (31, 32). Exponentially growing cells at A_{600} of 0.5, either in YPG or YPD, were washed with 10 mM potassium phosphate buffer, pH 7.0, and incubated for 30 min in the same buffer with 10 μM H_2DCFDA (dissolved in dimethyl sulfoxide). H_2DCFDA-loaded cells were incubated with 20 mM menadione as indicated. Cells were then washed, resuspended in distilled water, and disrupted using glass beads. Cell extracts (100 μl) were mixed in 1 ml of distilled water, and fluorescence was measured with λ_{EX} = 490 nm and λ_{EM} = 519 nm using a Shimadzu RF 5000 spectrofluorimeter. The value of λ_{EM} = 519 nm was normalized by protein concentration.

Measurement of Lipid Peroxidation—Detection of thiobarbituric acid reactive substances (TBARS) was carried out by means of a fluorimetric assay. Values were referred to a standard curve using malondialdehyde bis(dimethyl acetal) ranging from 0 to 0.25 nmol (33).

Sequence Analysis—Sequence analysis was carried out using the BLAST 2.0 program. Accession numbers were: for E2 subunit of yeast PDH, GI6324258; for E2 subunit of *E. coli* PDH, GI434011; for E2 subunit of yeast αKGDH, GI171783; for E2 subunit of αKGDH, GI43022; for yeast heat-shock protein 60, GI171719; and for *E. coli* DNA K, GI145773.

RESULTS

Hydrogen Peroxide Stress—Exponentially growing cultures of *S. cerevisiae* under fermenting conditions with glucose as carbon source (YPD) or under respiring conditions with glycerol (YPG) were challenged with hydrogen peroxide for 45 min. They were subsequently analyzed for protein oxidative damage, cell viability, and lipid peroxidation. As shown in Table I, basal levels of protein carbonyl content in crude extracts of cells grown in YPG were 1.6 times higher than those in cells grown in YPD. By contrast, there was a marked increase in protein damage after hydrogen peroxide treatment in cells grown in YPD, whereas those growing in YPG only showed a moderate increase in protein carbonyls. These results are consistent with a higher loss of cellular viability after the stress on cells in fermentative metabolism (treatment with 5 mM hydrogen peroxide resulted in 18% survival on YPD compared with 48% survival on YPG grown cells).

Pretreatment of cells with low doses of oxidant allowed them to tolerate higher doses of the compound (9, 10). To analyze protein carbonyl content in such preadaptation experiments, cells grown in YPD medium were treated for 30 min with 0.25 mM hydrogen peroxide before they were challenged with 5 mM hydrogen peroxide for 45 min. As shown in Table I, the carbonyl content was 0.85 nmol of carbonyl/mg of protein, a value clearly below the 1.58 for nonpretreated cells. This lower in-

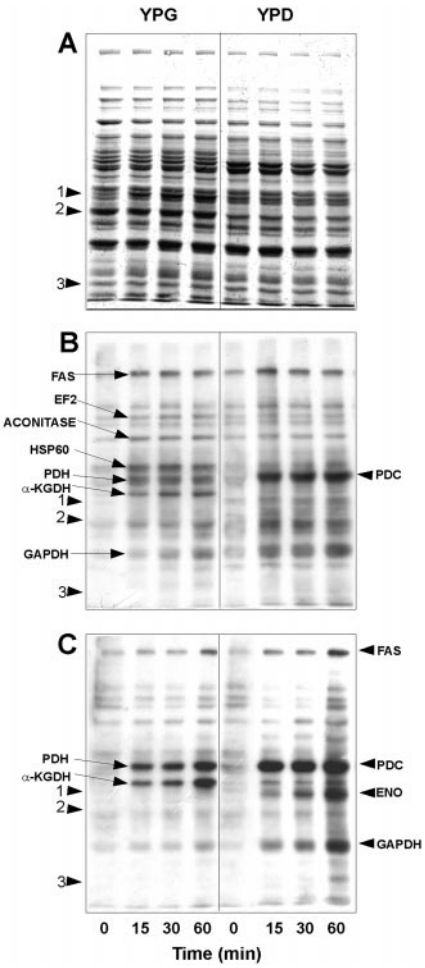

FIG. 1. **Pattern of oxidatively damaged proteins of *S. cerevisiae* after hydrogen peroxide or menadione stress.** Cells grown on YPG or YPD were taken at mid exponential phase and treated with 2 mM hydrogen peroxide or 20 mM menadione. Samples were taken at the indicated times and prepared as described under "Experimental Procedures." The protein stain is shown in *A*. Major oxidatively damaged proteins are indicated on anti-2,4-dinitrophenol-immunostained panels: hydrogen peroxide stress (*B*) or menadione stress (*C*). *FAS*, fatty acid synthase; *EF2*, elongation factor 2; *HSP60*, heat-shock protein 60; *PDC*, pyruvate decarboxylase; *ENO*, enolase and *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase. Numbers in the protein stain panel correspond to nonoxidized proteins: 1) 60 S ribosomal protein L3, 2) alcohol dehydrogenase II (zinc-dependent), and 3) phosphoglycerate mutase.

crease may be because of antioxidant defense systems induced under the preadaptation step. This result was consistent with a higher cell viability, which increased to 68% compared with 18% of the nonpretreated cells.

Fig. 1*B* shows the time course for increased protein damage resulting from exposure to 2 mM hydrogen peroxide in cultures grown under respiratory (YPG) and fermentative (YPD) conditions. Protein staining is shown in Fig. 1*A*. Identification of the main oxidized proteins was carried out as described under "Experimental Procedures." It can be observed that when hydrogen peroxide stress was performed in YPG-growing cells, the following mitochondrial proteins were selectively detected as major targets for oxidative damage: dihydrolipoamide acetyltransferase (E2 subunit of PDH), dihydrolipoamide succinyltransferase (E2 subunit of α-KGDH), and aconitase, all of them involved in energy production, and the chaperonin heat-shock protein 60. Among the cytosolic proteins the following were detected: glyceraldehyde-3-phosphate dehydrogenase, involved in glycerol metabolism; elongation factor 2, participating in the protein biosynthetic machinery; and the α-subunit of

TABLE II
Enzymatic activity remaining after oxidative stress

In the middle of the exponential phase, yeast cells grown aerobically on YPG or YPD were challenged for 45 min with 2 mM H_2O_2 or 20 mM menadione. Cell extracts or mitochondrial preparations were obtained to measure enzyme activities as described under "Experimental Procedures." The table shows the percentage of remaining activity with respect to control conditions (set as 100%). On YPG growing cells, control values, given in μmol/min/mg of protein, were: 0.12 for fatty acid synthase, 0.39 for aconitase, 0.68 for pyruvate decarboxylase, 0.29 for PDH, 0.13 for α-KGDH, and 7.66 for glyceraldehyde-3-phosphate dehydrogenase. For YPD growing cells, they were 0.11 for fatty acid synthase, 1.38 for pyruvate decarboxylase, 2.7 for enolase, and 4.72 for glyceraldehyde-3-phosphate dehydrogenase. Values given are the mean of at least three independent experiments with a variation of 5%.

Enzymes	YPG		YPD	
	H_2O_2	Menadione	H_2O_2	Menadione
Fatty acid synthase	80	7	41	30
Aconitase	25	10		
Pyruvate decarboxylase	100	41	41	12
PDH	41	15		
α-KGDH	16	7		
Enolase	96		91	56
Glyceraldehyde-3-phosphate dehydrogenase	84	65	53	53

fatty acid synthase. Table II shows the remaining enzyme activity values of fatty acid synthase, aconitase, PDH, α-KGDH, and glyceraldehyde-3-phosphate dehydrogenase after hydrogen peroxide treatment. The degree of inactivation was different for each enzyme and ranged from 16% for glyceraldehyde-3-phosphate dehydrogenase to 75% for aconitase and 84% for α-KGDH.

Under fermentative conditions (YPD medium), pyruvate decarboxylase was the main oxidized protein by the stress. The rest of the immunodetected proteins were the same as those presented in YPG medium, although the mitochondrial ones, synthesized in smaller amounts in these growth conditions, were almost undetectable. Fatty acid synthase and pyruvate decarboxylase activity levels diminished at around 60% as did that of glyceraldehyde-3-phosphate dehydrogenase, at around 50% (Table II).

Lipid peroxidation values measured under this stress condition are shown in Table I. There was a clear increase of similar magnitude in TBARS, in both, YPG and YPD, culture conditions.

Menadione Stress Conditions—Menadione, a redox-cycling compound, can be reduced *in vivo* to semiquinone, which in turn is able to reduce oxygen to superoxide anion radical, regenerating the oxidized quinone (34). To study the effect of this compound, different concentrations of menadione were added to yeast cultures growing either with glycerol or glucose as a carbon source. In both cases, the addition of the stressing compound caused growth arrest at concentrations above 0.5 mM for YPD and above 1 mM for YPG. The results presented in Table III indicate that, with respect to basal levels, menadione treatments produced higher amounts of protein carbonyl content in cells grown on YPD than in cells grown on YPG. In terms of cell viability, these results were in accordance with the higher stress tolerance of YPG grown cells, especially when the dose of menadione employed was 5 mM or greater (0.1% survival on YPD and 67% on YPG after 40 mM menadione). The results obtained in preadaptation experiments are summarized in Table III. Protein carbonyls showed a moderate increase (0.98 nmol/mg protein), which is clearly below the value of 1.32 obtained without preadaptation. In accordance with these results, viability of pretreated cells increased to 2.66%, a value significantly higher than the 0.1% obtained for nonpretreated cells.

Measurement of TBARS in cells stressed by incubation with 20 mM menadione revealed a clear increase, with respect to untreated cells, both in YPD (5-fold) and YPG (2-fold) grown

TABLE III
Carbonyl content of crude extracts and cell viability after exposure to menadione stress conditions

Cell viability is the percentage of the corresponding YPG and YPD control cultures and is the mean value of three independent experiments with a variation of ±5%. Values of carbonyl content (given in nmol of carbonyl/mg of protein) were determined in exponentially YPD and YPG growing cells (control conditions) and after 45 min of treatment with the indicated menadione concentration. The values summarized here are mean values for three separate experiments with a variation of ±0.05. TBARS values are given in nmols/mg protein with a variation of ±0.01.

Culture conditions	Survival	Carbonyl content	TBARS
mM			
YPD control	100	0.53	0.040
1	93	0.70	
5	67	0.78	
20	8	1.21	0.200
40	0.1	1.32	
40 (pretreated with 1 mM)	2.66	0.98	
YPG control	100	0.80	0.045
1	92	0.85	
5	90	1.10	
20	79	1.32	0.080
40	67	1.82	

cells (Table III). The differences in lipid peroxidation after this stress depended on the metabolic conditions of the cell and were supported by data on peroxides generated inside the cell measured with the fluorescent probe H_2DCFDA (Fig. 2). The intensity of fluorescence throughout menadione stress indicated that peroxides present in cells grown in YPD medium were always about 3–4-fold the values observed in YPG grown cells. The initial value in YPD grown cells was about 80% of that obtained in YPG (not shown).

The pattern of damaged proteins is presented in Fig. 1*C*. Two proteins became highly oxidized by menadione treatment in YPG grown cells. The NH_2-terminal sequence identified them as the E2 subunits of α-KGDH and PDH, which are structurally and catalytically similar multienzyme complexes, both involved in the oxidative metabolism. This strong oxidation would explain the low activity values obtained for both enzymes (Table II). Although a minor degree of carbonylation was observed, enzymes such as fatty acid synthase and aconitase also became largely inactivated (Table II). This would indicate that a mechanism different from those associated with carbonyl formation is responsible for the inactivation of these enzymes. From these data it is clear that both tricarboxylic acid cycle and electron transfer to the respiratory chain might be impaired. To determine whether stimulation of respiratory chain could be deleterious to the cell, cultures were treated with 40 mM menadione in the presence of 1% succinate, a compound able to reduce coenzyme Q. Under this experimental condition, a more rapid decrease in cell viability was observed when compared with the values obtained without succinate (Fig. 3). The addition of succinate alone had no effect on cell viability (not shown). In YPD grown cells, the major immunodetected bands after menadione stress were fatty acid synthase and three enzymes involved in glucose fermentation: pyruvate decarboxylase, enolase, and glyceraldehyde-3-phosphate dehydrogenase (Fig. 1*C*).

Modification of Lipoamide of α-KGDH and PDH—The importance of the oxidative inactivation of α-KGDH and PDH in energy metabolism prompted us to investigate their possible structural modifications, to better understand their inactivation. One possible explanation for this observed loss of enzyme activity may be the modification of lipoamide, the covalently linked form of dihydrolipoic acid, which is the common coenzyme for the E2 subunits of PDH and α-KGDH. Fig. 4 shows time course experiments with cultures subjected to menadione

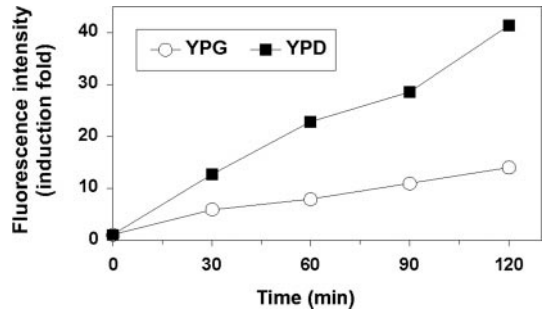

FIG. 2. **Intracellular peroxide levels produced under menadione treatment.** YPG grown cells loaded with 10 μM H_2DCFDA were treated with 20 mM menadione for the indicated times, and intracellular peroxides were measured as induction of fluorescent intensity with respect to control values. Results are given as the mean of four independent experiments.

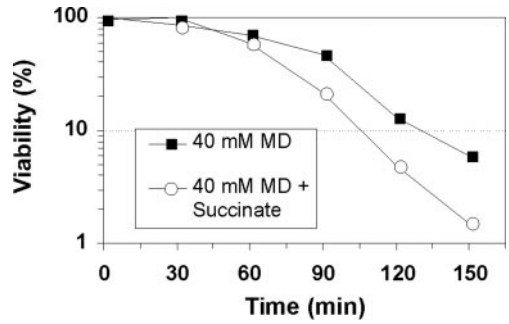

FIG. 3. **Effect of addition of succinate on menadione stress.** 1% succinate was added to exponential cultures of *S. cerevisiae* growing in YPG 15 min before stressing the cells with 40 mM menadione. Samples were taken to calculate cell viability at the indicated times.

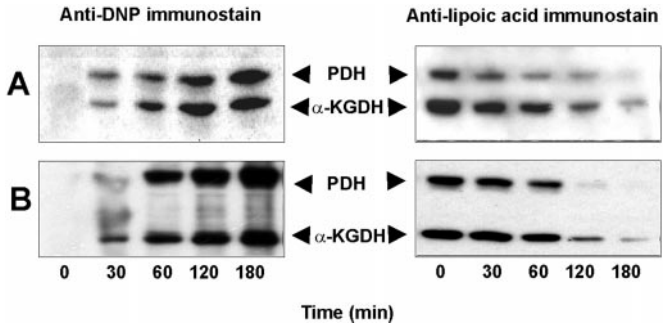

FIG. 4. **Comparative analysis of α-KGDH and PDH for protein carbonyl formation and lipoic acid recognition by specific antibodies.** Cells of *S. cerevisiae* grown in YPG (*A*) and *E. coli* grown aerobically in glucose (*B*) were submitted to superoxide stress with 10 mM menadione. At the indicated times, samples were taken and tested for protein carbonyls and lipoic acid recognition by Western blot.

stress, in which protein carbonyl formation and lipoamide presence in PDH and α-KGDH were analyzed. In *S. cerevisiae* (Fig. 4*A*), the increase in protein carbonyls in these enzymes correlated with a time-dependent decline in antibody recognition for their coenzyme, indicating a structural modification of lipoamide that, in turn, could have been responsible for their decreased enzymatic activity. To determine if such modification was a particular process of eukaryotic cells or if it is also present in prokaryotic cells such as *E. coli* when exposed to menadione stress, we reanalyzed and extended our previous observations in *E. coli* (5). Fig. 5 shows the protein oxidative pattern obtained when a culture of *E. coli* growing aerobically was stressed for 60 min with 10 mM menadione. From the four proteins outlined in the immunostained panel (Fig. 5*B*), we had already described two, EF-G (the eukaryotic counterpart of elongation factor 2) and the β-subunit of ATPase (5). In addi-

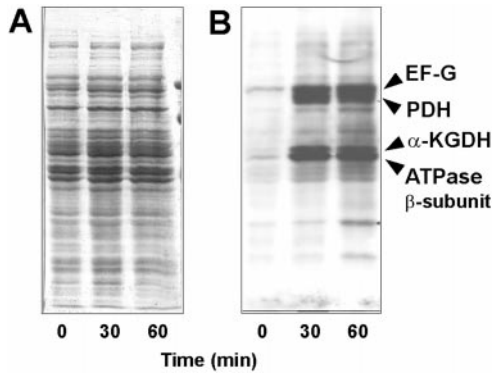

FIG. 5. **Menadione-induced protein oxidative damage in *E. coli*.** Aerobically growing *E. coli* cells were challenged with 10 mM menadione. At the indicated times, samples were taken and prepared for Western blotting. *A,* protein stain. *B,* anti-2,4-dinitrophenol immunostain. Identified proteins are indicated in *B*. *EF-G*, elongation factor G.

tion to these targets, we have now purified and identified (by NH_2-terminal sequencing), the E2 subunits of both PDH and α-KGDH as targets for oxidative damage. In both cases the enzymatic activities were almost undetectable after stress (data not shown). Interestingly, as for yeast, recognition of PDH and α-KGDH by the anti-lipoic acid antibody also decreased in a time-dependent manner in *E. coli* cells treated with menadione (Fig. 4*B*). This result strongly suggests that the same type of structural modification is occurring in both yeast and *E. coli* cells.

DISCUSSION

The objective of the current study was to identify oxidatively damaged proteins of *S. cerevisiae* subjected to oxidative stress. This would provide data to allow a better understanding of the mechanisms leading to energy depletion, growth arrest, and decreased cell viability and to compare these effects with those observed in *E. coli*.

It is known that the amount of mitochondria in cells grown in YPD is lower than in YPG and that the generation of ROS is therefore minor. Furthermore, many of the antioxidants defenses are repressed by glucose (catalases, superoxide dismutases, and glutathione peroxidase), and aerobic respiring cells display higher levels of such detoxifying enzymes (8). Bearing this in mind, it is conceivable that basal levels of protein carbonyl content obtained from cells growing under fermentative conditions would be lower than those exhibited under respiratory conditions. These considerations would also explain the smaller increase (with respect to basal levels) in protein carbonyls observed in YPG grown cells after hydrogen peroxide or menadione stress compared with those found in YPD grown cells (Tables I and III). Lipid peroxidation measured as TBARS showed a marked increase after oxidative stress conditions. Using hydrogen peroxide, no differences were observed between YPD and YPG, whereas with menadione, TBARS detected in YPG grown cells were significantly fewer than those detected in YPD grown cells. This may have been a consequence of the smaller amounts of ROS in YPG grown cells, compared with YPD grown cells (Fig. 2). All of these results support the viability data obtained, which indicated that *S. cerevisiae* cells grown in YPG are better prepared for coping with oxidative stress than those grown in YPD media. It is known that preadaptation of *S. cerevisiae* to low doses of stressing compounds induces a tolerance to higher doses (10, 35). Our data indicated that preadaptation-induced protection (measured as increased cell viability) agreed with reduced protein carbonyl content found after oxidative stress in pretreated cells in contrast to that exhibited by nonpretreated cells. Preinduction of antioxidant defense proteins such as superoxide dismutases, catalases, metallothionein, or glucose-6-phosphate dehydrogenase could account for the lower levels of carbonyls observed (8).

The patterns of protein oxidation presented in Fig. 1 indicate selectivity in the main targets, especially under menadione stress, which is probably because of the site-specific generation of ROS. This selectivity is highlighted by the fact that detection is not dependent on the amount of a particular protein, clearly demonstrated by the identification of major bands as nondamaged proteins. One of those is the isozyme II of alcohol dehydrogenase (Fig. 1), which displayed 90% of enzymatic activity after stressing the cells either with menadione or hydrogen peroxide (not shown). The maintenance of activity in enzymes with no significant carbonyl content also applies, under certain stress conditions, to pyruvate decarboxylase and enolase from cells growing on YPG treated with hydrogen peroxide (Table II).

Among the oxidatively damaged proteins, cytosolic and mitochondrial ones were found. In the first group it is interesting to mention the elongation factor 2, a translocase involved in the elongation step of protein synthesis in eukaryotic cells, which has also been described as oxidatively damaged in hepatocytes (37). In addition, its prokaryotic counterpart, elongation factor G, has been identified as a main target in *E. coli*, both in stressed cells (5) and in mutants lacking cytoplasmic superoxide dismutase (38).

Among glycolytic enzymes, oxidized glyceraldehyde-3-phosphate dehydrogenase and pyruvate decarboxylase were clearly detected under menadione and hydrogen peroxide stress, whereas enolase only appeared to be oxidized after menadione stress. Inactivation of these enzymes would slow down glycolisis and the tricarboxylic acid cycle. In such a case, as described by Godon *et al.* (12), glucose would be diverted to the pentose phosphate pathway, generating additional NADPH, which is needed by antioxidant enzymes. Glyceraldehyde-3-phosphate dehydrogenase, which catalyzes the conversion of glyceraldehyde 3-phosphate to 1,3-diphosphoglycerate coupled to the reduction of NAD to NADH, has a highly reactive thiol at its active center. Protein carbonyls detected in this enzyme can be explained on the bases of *in vitro* studies made by Uchida and Stadtman (39) demonstrating the inactivation of the enzyme by 4-hydroxynonenal. This lipid peroxidation product reacts, among other amino acids, with sulfhydryl groups of cysteine residues to form thioether adducts. The aldehyde function of 4-hydroxynonenal can react with DNPH to form a hydrazone derivative (39). We cannot rule out the possibility that the carbonylation of this enzyme could be because of amino acid side-chain modification resulting from thiol oxidation of the cysteine located at its catalytic site (40). In this context, it has been described that a modification of this enzyme by NADH is dependent on superoxide anions and mediated by thiols (41). Likewise, in the α-subunit of fatty acid synthase, the thiol group present in the phosphopantheine linked to the acyl carrier protein would seem to account for the carbonylation and a high degree of inactivation observed under superoxide stress conditions with menadione. According to this result, the β-ketoacyl-(acyl-carrier protein) synthase of *E. coli* was described as oxidatively damaged after starvation (42).

It is evident that in YPG grown cells, the major targets detected under superoxide stress conditions were the mitochondrial proteins, α-KGDH and PDH. Under hydrogen peroxide stress, these two proteins were also main targets, in addition to other mitochondrial ones, such as aconitase and the heat-shock protein 60. It is interesting to note that, in *E. coli*, another chaperone namely DNA K, was also clearly detected as a target under hydrogen peroxide stress (5). These results suggest that oxidation of these molecular chaperones may be a consequence

of acting as a shield for proteins protecting them from ROS.

In the trichloroacetic acid cycle, aconitase catalyzes the isomerization of citrate to isocitrate. Its prostetic group is an iron-sulfur cluster, which is prone to oxidation by superoxide anions releasing free iron (43), which, in turn, triggers oxidative damage to macromolecules (44). In this situation, aconitase, like α-KGDH, lost most of its activity (75–90%) and also became itself carbonylated. Major carbonyl content was detected in a peptide encompassing Val^{334} to Asp^{527}, which interestingly includes amino acid sequences around the iron-sulfur cluster (not shown). In accordance with this result, detection of protein carbonyls in aconitase has been described during the aging of the housefly (45). Although the strong inactivation observed does not correlate, apparently, with the low carbonylation detected by Western blot, this could be explained by modifications other than carbonyl formation, such as releasing iron from its cluster or by reaction with peroxynitrite (46).

αKGDH and PDH are multienzyme complexes, which catalyze the conversion of α-ketoglutarate to succinyl-CoA and pyruvate to acetyl-CoA, respectively. Each complex is composed of multiple copies of three subunits: E1, a thiamine pyrophosphate-dependent 2-oxo-acid dehydrogenase, E2, a dihydrolipoamide acyltransferase, and E3, a FAD-containing dihydrolipoamide dehydrogenase. Whatever the stressing conditions used, E2 from both PDH and αKGDH were immunodetected as heavily oxidized proteins. This result agrees with the decreased enzyme activity of these complexes and was coincident with the modification of their coenzyme, dihydrolipoamide. One possible explanation for the decline in the immunodetection of lipoic acid and the detected carbonyl groups would be a reaction with 4-hydroxynonenal, a lipid peroxidation product, which has been reported to form a Michael addition product (23, 47, 48).

Clearly, one major conclusion of the present work is the importance of arresting mitochondrial metabolism under oxidative stress conditions to prevent deleterious production of ROS. These species generated inside the cell by redox cycling of menadione, heavily target mitochondrial enzymes causing their inactivation and consequent energy depletion and growth arrest. Under such stress, the exogenous addition of succinate (which causes a stimulation of electron transfer to coenzyme Q (22)) increases the production of ROS, which, in turn, reduce cell viability (Fig. 3).

Compared with menadione, the exogenous addition of hydrogen peroxide would seem to produce a less selective pattern of protein damage. Results showed that, although more proteins were affected, major targets were mitochondrial ones. The susceptibility of mitochondrial proteins can be explained by the fact that low molecular weight Fe^{2+} complexes accumulate in mitochondria (36). Under stress situations, this will exacerbate the production of hydroxyl radicals by Fenton and/or Haber-Weiss reactions. This rationale, based on the special characteristics of mitochondrial metabolism, can also be applied to explain such phenomena as the toxicity of doxorubicin (a potent antitumor drug), tumor necrosis factor, and the elevated concentrations of Ca^{2+}, which stimulate ROS production by mitochondria (13). It would be interesting to know whether the modified proteins described in the present work could account for some of the toxic effects described in these cases.

Concerning selective oxidation of proteins, the results presented in this paper indicate that prokaryotic and eukaryotic cells display clear homologies. These homologies can be a consequence of their structural and/or functional relationship. In this context, sequence homologies of PDH and α-KGDH from *S. cerevisiae* with respect to those from *E. coli* were 40 and 59%, respectively. The homologies are even greater when their active sites or lipoic acid binding signatures are compared. This analysis would tend to suggest the importance of the protein structure on the selectivity of oxidative targets; nevertheless, in the case of heat-shock protein 60 and DNA K, they share no significant similarity, indicating that, in this case, chaperoning function could be a reason for their selective damage.

The question of whether such proteins have been selected as targets during evolution to better preserve the integrity of the cell after a stress situation remains to be determined.

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METABOLISM AND BIOENERGETICS:

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