Protein Oxidation in \textit{G}_0 Cells of \textit{Saccharomyces cerevisiae} Depends on the State Rather than Rate of Respiration and Is Enhanced in \textit{pos9} but Not \textit{yap1} Mutants* \\

Received for publication, February 27, 2001, and in revised form, June 18, 2001
Published, JBC Papers in Press, June 28, 2001, DOI 10.1074/jbc.M101796200

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Immunodetection of protein carbonyl groups demonstrates that growth arrest elicited by carbon or nitrogen starvation causes an increased oxidation of proteins in \textit{Saccharomyces cerevisiae}. Mutant analysis suggests that the response regulator Pos9p is involved in mitigating self-inflicted oxidative damages in \textit{G}_0 cells, whereas Yap1p is primarily required in growing cells. The data also suggest that oxidation of target proteins is not \textit{a priori} an effect of arrest of cell division or nutrient depletion and cannot be explained by the respiratory activity alone nor a high ratio of catabolic/anabolic activity in \textit{G}_0 cells. Instead, we observed that starvation elicits a transition in the respiratory state (from phosphorylating to nonphosphorylating respiration) and that this transition is associated with a stepwise increase in protein oxidation. During carbon starvation, this transition and increase in oxidation occurs immediately as the carbon source is depleted, growth is arrested, and the respiratory rate falls drastically. In contrast, during nitrogen starvation and excess carbon the respiratory state transition and stepwise increase in protein oxidation are markedly delayed and occur long after the nitrogen source has been depleted and division and growth-arrested. Oxidation in \textit{G}_0 cells could be enhanced by treating cells with low concentrations of antimycin A and attenuated with myxothiazol, indicating that protein oxidation is intimately linked to reactive oxygen species generated by semiquinones of the Q-cycle. Thus, the work presented suggests that the degree of coupling in the mitochondrial respiratory apparatus rather than the overall rate of respiration affects the degree of protein oxidation in nondividing yeast cells.

It has been proposed that aging results from random deleterious events, and oxidative damage has been suggested to be one major contributor to such stochastic degeneration of organisms and their cells. Denham Harman was perhaps the first to suggest that free radicals produced during aerobic respiration cause cumulative oxidative damage, resulting in aging and death (1). The theory gained in credibility with the identification of superoxide dismutase, which provided compelling evidence of \textit{in vivo} generation of superoxide anions (2). The hypothesis has later been supported by different experimental data demonstrating that levels of oxidatively damaged macromolecules, including DNA, proteins, and lipids, increase with age in all species examined so far and that oxidatively modified proteins lose their catalytic activity and structural integrity. In addition, support of the theory comes from experiments in which the life span of fruit flies was prolonged by overproducing antioxidants (3), and recent identification of gerontogenes (genes whose alteration causes life extension) in \textit{Caenorhabditis elegans} further supports the notion of a strong correlation between longevity and oxidative stress defense. In addition, the life span of resting unicellular microbes, such as \textit{Escherichia coli} (4) and \textit{Saccharomyces cerevisiae} (5, 6), appears to be limited by the cell’s ability to combat reactive oxygen species (ROS).

The causal factors behind the increased levels of oxidized macromolecules in resting and aging cells is a key question that has not been resolved. Some attempts have been made to correlate oxidation in aging cells with a reduced activity (or abundance) of the antioxidant defense and repair systems (or proteases involved in degradation of oxidized proteins) (6). However, these attempts have generated conflicting results. For example, catalases have been demonstrated to either increase or decrease with age depending on the tissues analyzed (7), and in other studies it has been demonstrated that some antioxidant defense proteins may increase, while others decrease, with age in the same tissues (8). Moreover, as pointed out by Beckman and Ames (9), it is not clear whether an elevated abundance of an antioxidant defense system in any given cell indicates that there will be less oxidative damage in this cell or that it is experiencing an increasing oxidative load. In procaryotic model systems, such as \textit{E. coli}, it is known that the levels of both primary and secondary oxidative defense proteins increase markedly during growth arrest and that the population becomes increasingly resistant to external oxidative stresses (10, 11). Yet, the level of oxidatively damaged proteins in such a resting \textit{E. coli} population increases (4, 12).

Apart from these difficulties, the notion that increased oxidation in aging cells is due to a diminished ability of the defense systems to counteract such oxidation raises the ques-

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* This work was supported by a grant from the Swedish Natural Science Research Council (to T. N.), by the commission of the European Union via Contract BIO4-CT98-0562, and by a Chalmers Bioscience Initiative (to L. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: ROS, reactive oxygen species; CCCP, carbonyl cyanide m-chlorophenylhydrazone; TET, triethyltin bromide; CBS, central bureau seer; RSV, respiratory state value; PS3, pseudostate 3; PS4, pseudostate 4; CoQ, semiquinone; CoQH₂, reduced quinones; CoQ, oxidized quinones; GSH, reduced glutathione; GSSG, oxidized glutathione; H₂O₂, hydrogen peroxide; H₂O₂, reduced glutathione; CoQH, reduced quinones; CoQ, oxidized quinones; O₂⁻, superoxide ion; PVDIF, polyanhydride difluoride; CoQH₂, semiquinone; CoQH, reduced quinones; CoQ, oxidized quinones; O₂⁻, superoxide ion; PVDIF, polyanhydride difluoride; 1, liter; DNP, 2,4-dinitrophenolhydrazone.
tion of why such systems then fail to function in resting or old cells. The suggestion that this is primarily due to free radicals and oxidative damage is, of course, a circular argument. Two alternative possibilities are that (i) the respiratory apparatus, or other enzymatic activities, has an increased tendency to generate ROS during growth arrest or that (ii) the levels of oxidized proteins increase in resting and nongrowing cells simply due to the fact that such cells have an ongoing respiratory activity but little or no ability to dilute damaged proteins with sufficient levels of de novo macromolecular synthesis. The latter model thus points to the possibility that the lack of self-replacement in a metabolically active aerobic system causes an unavoidable increase in oxidatively damaged cell constituents. In this work, we have approached these possibilities by using G₀ cells of S. cerevisiae as a model system for studying oxidation in cells entering a resting state. We established that these cells could be used to study stasis-induced oxidation by demonstrating that the level of carbonylated proteins markedly increases as a result of starvation-induced growth arrest in S. cerevisiae similar to starving E. coli cells and aging eukaryotes. The response regulator, Pos9p, appears to be specifically involved in reducing self-inflicted oxidation in G₀ cells, whereas the Yap1p regulator is more important during growth. In addition, we used different batch and fed-batch cultivation systems in combination with on-line measurements of metabolic activities to show that oxidation of proteins is not a priori caused by growth arrest and a high respiratory activity in nongrowing cells. Nor is oxidation an immediate effect of the lack of a growth-supporting nutrient. Instead, we found that a shift in the state of respiration to one in which the mitochondrial ATPase is no longer working during stasis is intimately associated with an increase in the oxidation of target proteins. This is the first demonstration that state transitions occurs in vivo during growth arrest and that such transitions are associated with increased oxidative damage. In summary, our data favor the model that stasis increases the tendency of the respiratory apparatus to generate ROS and that this tendency may be linked to stasis-induced alterations in the functioning of the oxidative phosphorylation. We discuss how such a reduced phosphorylation may generate ROS in view of existing data concerning respiratory state transitions in isolated mitochondria.

EXPERIMENTAL PROCEDURES

Strain and Cultivation Media—The strains used in this work are listed in Table I. All experiments presented in this work have been performed with a central bureau seer (CBS)-based minimal medium. The medium used was designed differently depending on the starvation conditions as follows: carbon starvation medium; glucose (5 g/l), ammonium sulfate (5 g/l), potassium dihydrogen phosphate (3 g/l), and magnesium sulfate (0.5 g/l). The nitrogen starvation medium was the same as carbon starvation medium, but 10 g/l glucose and 0.12 g/l ammonium sulfate was used. The phosphate starvation medium contained glucose (10 g/l), ammonium sulfate (5 g/l), potassium dihydrogen phosphate (66 mg/l), potassium chloride (1.6 g/l), and magnesium sulfate (0.5 g/l). The fed-batch medium (pumped medium) contained ethanol (12.5 ml/l), potassium dihydrogen phosphate (3 g/l), and magnesium sulfate (0.5 g/l). All media were supplemented with 1 ml/l of a vitamin solution and 1 ml/l of a trace elements solution. The vitamin solution contained biotin (0.05 g/l), calcium pantothenate (1.00 g/l), nicotinic acid (1.00 g/l), myo-inositol (25.0 g/l), thiamin hydrochloride (1.00 g/l), pyridoxol hydrochloride (1.00 g/l), and p-aminobenzoic acid (0.20 g/l). The trace elements solution contained EDTA (15 g/l), zinc sulfate heptahydrate (4.5 g/l), manganese chloride tetrahydrate (1.0 g/l), cobalt (II)-chloride hexahydrate (0.3 g/l), copper (II)-sulfate pentahydrate (0.3 g/l), disodium molybdenum dihydrate (0.4 g/l), calcium chloride dihydrate (4.5 g/l), iron sulfate-heptahydrate (3.0 g/l), boric acid (1.0 g/l), and potassium iodide (0.1 g/l). Analysis of mutants was performed in 100-ml cultures contained in 1-l flasks shaken at 200 rpm at 30 °C. Growth medium of auxotrophic strains (see Table I) was similar to the carbon starvation medium but contained 1 g/l of glucose (so that cell density never constituted a limitation for oxygen diffusion), leucine (25 mg/l), uracil (9 mg/l), histidine (9 mg/l), and methionine (6 mg/l) and was buffered with 100 mM potassium hydrogen phthalate and NaOH at pH = 5. All growth experiments of prototrophic strains that were not subject to specific treatments with drugs (see "Treatments with Drugs") were performed in a 3-l fermentor during controlled conditions. Temperature was kept at 30 °C, and pH was constantly adjusted (between 4.9 and 5.1) by addition of 1 M solutions of NaOH and HCl. Central palettes fixed on a rotor operated the stirring at a speed of 500 rounds/min. Sterile air was continuously pumped into the fermentor at a rate of 750 ml/min so that oxygen never limited growth.

Colonizing Forming Unit Counting—50 μl of serial dilutions of cultures were plated onto VPD plates (1% yeast extract, 2% glucose, 2% peptone,
2% agar). After 2 days of incubation at 30.0 °C, colonies were counted from plates that harbored between 30 and 300 colonies.

**Treatments with Drugs**—Cells submitted to specific treatments were removed from the fermentor and placed into temperature-controlled flasks (in a water bath). The volume of the culture was 1/10 of the flask volume, and the stirring was operated with magnetic bars at 500 rpm. The pH-controlling system of the fermentor was turned off to monitor the pH change. Moreover, the pH, which was checked at the end of the cultures in every flask, remained constant. For each sample analyzed, a respiration titration curve was made to determine the optimal concentration for either inhibition (TET) or activation (CCCP) of respiration. The range of the concentrations used during starvation conditions (regardless of the limiting nutrient) was from 1.0 to 10 μg of CCCP (Sigma)/mg dry weight and from 15 to 30 μg of TET (Aldrich)/mg dry weight. During exponential phase, the concentrations needed for optimal effect were 1 μg of CCCP/mg dry weight and 15 μg of TET/mg dry weight TET. Concentrations of antimycin A (Sigma) and myxothiazol (Fluka) were always 15.0 μg/mg dry weight and 10.0 μg of myxothiazol/mg dry weight, respectively.

**Microcalorimetry**—The heat production rate (dQ/dt) was measured with a heat conduction-type multichannel microcalorimeter (Bioactivity Monitor LKB 2277; Thermometric AB, Järfälla, Sweden) equipped with flow-through cells as described by Larsson et al. (13). The microcalorimeter was operated at 30.0 °C at a measuring range of 900 micro-watts. The cultures were pumped at a rate of 75 ml/h from the growth vessel by a peristaltic pump as described previously. Calibrations were
Protein Oxidation in Growth-arrested S. cerevisiae

Protein Oxidation Increases in \( G_0 \) Cells and Is Enhanced in pos9 Mutants—The carbonyl content of total proteins was measured immunochemically, and densitometric quantification of the blots demonstrated a severalfold increase in carbonyl content in wild-type yeast cells as a result of arrest of proliferation during starvation (Fig. 1). We then elucidated whether any of the known systems involved in the defense against external oxidative agents (18–21) also have a role in counteracting self-inflicted oxidative damage elicited by growth arrest. As shown in Fig. 2, a mutation in YAP1, encoding a B-ZIP transcription factor required for resistance against hydrogen peroxide (19, 22, 23), resulted in significantly higher levels of carbonylation in growing cells, but the levels of oxidized proteins were subsequently reduced during growth arrest. In contrast, a mutation in the response regulator gene, POS9, caused an elevated carbonylation only in growth-arrested cells (Fig. 2). Specifically, the reduction in oxidized protein levels observed following the initial oxidation peak in wild-type cells was not detected in mutants lacking Pos9p (Fig. 2). Thus, it appears that the roles of Yap1 and Pos9 in the defense against endogenously generated oxidative damage are distinctive and growth phase-dependent.

Protein Oxidation in \( G_0 \) Cells Occurs Concomitantly with a Drastic Reduction in the Rate of Respiration—We asked whether the oxidation elicited by growth arrest was correlated to some specific alterations in metabolic activity. To approach this we measured, on-line, total metabolic activity (heat production), oxygen consumption, and carbon dioxide production in parallel to the immunochemical analysis of protein carbonyls. We used batch cultures in a medium designed so that carbon was exhausted first causing cells to enter the \( G_0 \) phase (see “Experimental Procedures”). The growth of \( S. \) cerevisiae in the medium employed can be divided into several phases. In the first phase, glucose is mainly fermented to ethanol via the Embden-Meyerhof-Parnas pathway even though O2 is not limiting. This is followed by a diauxic shift in which the catabolism is shifted to ethanol utilization via mitochondrial respiration (respiratory phase, Fig. 1A). Cell proliferation continues in this phase albeit at a reduced rate. When ethanol is fully consumed (carbon depletion), the cells stop dividing and enter the “stationary phase”. At the time of carbon depletion, the total metabolic activity (recorded as the heat production rate) drops precipitously (Fig. 1A and B). The carbonylation assay demonstrated that protein oxidation increases rapidly and peaks following carbon depletion (Fig. 1C). The level of carbonylated proteins was then repressed gradually (Fig. 1C). However, the carbonyl levels never reached the low levels observed in the exponential growth phases. We next conducted a similar experiment with a medium designed in such a way that nitrogen or phosphate was the first nutrient to become limiting (see “Experimental Procedures” and Fig. 3 (phosphate limitation not shown)). In these experiments, nitrogen (or phosphate) was incubated with primary antibody, specific to the DNP moiety of the proteins, and subsequently incubated with a secondary (IgG goat anti-rabbit) horseradish peroxidase-antibody conjugate directed against the primary antibody. Filters were then treated with chemiluminescence blotting substrate (horseradish peroxidase) for detection. The blots were exposed to autoradiographic film that was subsequently developed. Exposition time could vary from 5 to 60 s. All direct comparisons are, therefore, made on data taken from the same filter.

Denatometry—Quantitative analysis of protein samples analyzed for carbonyl groups content was performed by densitometry using the Scion Image software (Scion Image Beta 4.02 Win). This software is based on the Macintosh NIH Image Software.

Reproducibility—All experiments presented in this paper have been repeated at least three times to confirm reproducibility. Representative results are shown in the figures.

RESULTS

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depleted subsequent to the respiro-fermentative phase and the diauxic shift. During nitrogen depletion we observed that the optical density of the culture increased despite the fact that cell division had stopped (measured by colony-forming unit counting (Fig. 3A). As seen in Fig. 3B, the rate of respiration remains relatively high (consumption of ethanol) even when proliferation is blocked due to nitrogen depletion. Yet, the protein carbonylation level increases only slightly during this phase, and the rapid peak seen during carbon starvation was not elicited by nitrogen starvation (Fig. 3B). However, as soon as carbon (ethanol) was totally depleted and the respiration rate dropped (arrow 2 in Fig. 3A), carbonylation peaked in a similar fashion to that observed for carbon starved cells depicted in Fig. 1, A–C. Based on these experiments, we conclude that protein oxidation in G₀ cells is not a result of proliferation arrest per se. In contrast, elevated oxidation of target proteins occurs when the increase in optical density is arrested and the rate of respiration falls precipitately concomitantly with the depletion of the carbon source. Next, we wanted to distinguish which of these factors was the critical one in eliciting elevated oxidation.

Protein Carbonylation during the G₀ Phase Is Not the Immediate Result of Starvation or Growth Arrest—To separate the observed drop in metabolic activity (recorded as heat production and respiration rate) from the depletion of the carbon source, we applied a fed-batch cultivation system. The medium pumped into the fermentor of the nitrogen starved cells contained ethanol as the sole carbon source. The medium was designed in such a way that nitrogen was exhausted first in the culture (for detail, see “Experimental Procedures”), and before ethanol was fully depleted, we pumped a medium with ethanol containing no nitrogen into the fermentor as described (see “Experimental Procedures”). The pump speed was 9.6 ml/h and corresponded to half the rate of ethanol consumption from cells grown under these conditions (taking into account the ethanol concentration of the medium; see "Experimental Procedures"). As a consequence, the metabolism of the cells decreased slowly due to the nitrogen limitation, but carbon was always present in the medium (Fig. 4A). Under the conditions employed, the levels of carbonylated proteins remained relatively constant for about 70 h after the nitrogen was depleted and growth arrested. After this time, however, carbonylation increased in a relatively rapid and stepwise fashion (Fig. 4B). We could not detect any discontinuity in either the global metabolic activity or respiration at the time of the increase in protein oxidation, which occurred long after proliferation and growth had been arrested. Indeed, the oxygen consumption rate was very low

Fig. 6. Protein carbonylation and respiratory state values in nitrogen-starved, carbon-sufficient G₀ cells. Cells were grown in fed-batch culturing conditions as described in the legend to Fig. 4. The upper panel (A) depicts optical density at 610 nm and oxygen consumption rate. The lower panel (B) shows respiratory state values (open squares) and carbonylation (filled squares). Samples were taken and split in two batches. One was used to analyze the respiratory state as described in the legend to Fig. 5, while the other was subjected to the carbonylation assay.

Fig. 5. Experimental approach to determine the in vivo RSV. A, cells were removed from the fermentor and injected immediately into a high performance oxygraph. The oxygen concentration in the cuvette was then measured. Subsequently, TET was added, and the oxygen consumption rate obtained during this block of ATPase activity is called a pseudostate 4 (PS4). The inhibition of ATPase mimics ADP depletion in a typical state 4 respiration obtained on isolated mitochondria. The oxygen consumption rate obtained after subsequent addition of CCCP is defined as a pseudostate 3 (PS3). As a control, a glucose/ethanol solution was added after the addition of the drugs to confirm that the oxygen consumption rate measured in the presence of CCCP was not limited by substrate availability. B, the derivative curve of the oxygen concentration (the oxygen consumption rate) is shown in the lower panel. To assign a RSV to the oxygen consumption rate initially measured (α), the formula 100 \((1 - \alpha) + \gamma - \beta\) was used. β is the oxygen consumption rate measured after addition of TET, and γ is the oxygen consumption rate measured after addition of CCCP. Thus, a total failure of TET to inhibit oxygen consumption gives a state value of 0 and indicates a PS4-type respiration, while a total absence of effects using CCCP indicates a PS3.
activity has reached an extremely low level. During carbon starvation this drop in activity occurs immediately and precipitately (Figs. 1B and 3B), whereas in nitrogen-starved, carbon-sufficient cells, this low activity is reached after a slow and more gradual reduction in metabolic activity (Fig. 4).

The Stepwise Increase in Protein Oxidation in G0 Cells Is Correlated with a Transition in the Respiratory State—According to several studies performed on isolated mitochondria (24, 25), the ROS are mainly produced by mitochondria. Korshunov et al. (24) showed that, in isolated mitochondria, ROS production was tightly correlated to the electrical component of the mitochondrial inner membrane proton-motive force. In addition, Fitton et al. (26) showed that in isolated yeast mitochondria, the membrane potential changes significantly with the respiratory state (26). It had also been shown that respiratory rate decreased in stationary phase cells and that treatment with antimycin A could enhance superoxide production in vivo (6). However, the occurrence of state transitions in vivo and whether they are associated with an increased oxidative damage have remained open questions. The original definitions of the respiratory states were not meant to describe in vivo physiological conditions (27), but it is possible to define “pseudostates” (PS) of intact cells using different inhibitors and therefore to indirectly assess the membrane potential in vivo. In this study, we used TET, a lipophilic ATPase inhibitor (28), and CCCP, a common protonophore to elucidate the PS of intact yeast cells. Despite the fact that TET has been described as a potent inhibitor of different enzymes such as hexokinase (29), the concentrations (2–6-fold less than the concentrations used to see an effect on hexokinase) used here and the short time span of the experiments exclude such effects. The cells were sampled and analyzed immediately in a high performance oxygraph (see “Experimental Procedures” for details). The first oxygen consumption rate measured (d[O2](TET)/dt) was called a (Fig. 5B). TET was then added to mimic ADP depletion (28, 30), the consequent oxygen consumption rate (d[O2](TET)/dt) measurement was called β (Fig. 5B). This value corresponds to a pseudostate 4 (PS4 on Fig. 5A). It was then possible to reverse the inhibition of TET on the respiratory rate by adding CCCP. The cells then showed an oxygen consumption rate (d[O2](CCCP)/dt) called γ (Fig. 5B). This value corresponds to a pseudostate 3 (PS3 on Fig. 5A). One could argue that the respiration rate with CCCP present would be very different from a real state 3, but it turned out that during exponential growth, respiration was almost not stimulated at all by CCCP, demonstrating that the approximation was reasonable. To give a RSV corresponding to α, the following formula was used.

\[
RSV = \frac{d[O_2](TET)}{d[O_2](CCCP) - d[O_2](TET)} \times 100 - \frac{\alpha - \beta}{\gamma - \beta} \times 100 \quad (Eq. 1)
\]

As mentioned above, during exponential growth, the RSV was indistinguishable from a PS3 and therefore had a value of ~100 (Fig. 6). As the culture entered nitrogen starvation, the respiratory rate decreased significantly, and the RSV dropped to 40 (i.e. a value approaching a PS4; Fig. 6). It is interesting to note, however, that respiration could still be inhibited with TET and activated with CCCP. Therefore, a true control is exerted by the oxidative phosphorylation for prolonged periods of starvation. For a period of ~50 h after the entry into nitrogen starvation, the state value remained constant despite the fact that the rate of respiration decreased progressively. After ~100 h of incubation, TET could no longer inhibit the respiratory rate, and the RSV was therefore 0 (i.e. a PS4; Fig. 6). The transition to a state value of 0 occurred concomitantly with the stepwise increase in protein oxidation (Fig. 6), suggesting that those two phenomena may be linked in a cause-effect manner. Moreover,
The Accumulation of Semiquinones Increases Protein Oxidation in Vivo—To approach the question of whether the semiquinones are involved in the oxidation of proteins observed in G0 cells, we repeated the fed-batch experiment described above. After 70 h of incubation, the pumping of medium was stopped, and three times 100 ml of the culture were transferred to three E-flasks. One flask served as a control and the two other contained antimycin A and myxothiazol, respectively. Both these drugs affect the Q-cycle and inhibit respiration. Antimycin A blocks the quinones in their radical form (CoQ\(_2\)), whereas myxothiazol causes the accumulation of the reduced form (CoQH\(_2\)). In addition, it is known that both these drugs decrease the membrane potential (31). We used concentrations of the drugs such that respiration was inhibited to the same extent and determined the effects on protein carbonylation compared with the nontreated control (Fig. 10). Myxothiazol attenuated protein oxidation, whereas antimycin A enhanced carbonylation significantly. The pH remained between 4.9 and 5.3 during the experiment in all flasks analyzed. This experiment favors the hypothesis according to which the redox state of the quinones is affecting the level of protein oxidation via the production of the superoxide ion.

**DISCUSSION**

*S. cerevisiae* has been used as a model system for studying two different aspects of cellular senescence. One aspect concerns replicative senescence, *i.e.* the mandatory loss of replicative ability after a certain number of successive doublings, which, in yeast, appears to be dictated by genome instability, specifically in the ribosomal DNA (32). The other aspect concerns the loss of reproductive ability of cells subjected to growth arrest in stationary phase. It has been suggested that such stationary phase yeast cells may be a model system for aging of somatic cells of higher eukaryotic multicellular organisms (5, 6). This argument is based on the fact that stationary phase yeast cells may be a model system for aging of somatic cells of higher eukaryotic multicellular organisms (5, 6). This argument is based on the fact that stationary phase yeast cells resemble somatic cells in two important aspects: (i) they have exited the cell cycle and entered the G0 phase, and (ii) most of their energy comes from mitochondrial respiration (5). The life span of such G0 cells has been suggested to be intimately linked to mitochondrial respiration and cellular management of ROS (5, 6), whereas the effect of ROS on replicative senescence remains a controversial issue (33, 34).

The role of oxidative damage leading to loss of viability of G0 cells is based upon results demonstrating that mutant cells lacking either copper-zinc superoxide dismutase, manganese superoxide dismutase, or both lost culturability at an accelerated rate in stationary phase (5, 6). This is similar to results obtained with superoxide dismutase mutants of *E. coli* (12, 35). However, from these results we cannot make the assumption that the life span of wild-type cells is restricted by oxidative damage; they only demonstrate that superoxide dismutase mutants are worse off in stationary phase than their wild-type counterpart. In this paper, we approached this problem by directly measuring oxidative damage to proteins in wild-type yeast cells entering the G0 phase due to nutrient starvation. We used an immunological assay for the detection of protein carbonylation, which is an irreversible and highly deleterious oxidative modification of proteins (36). As demonstrated, we obtained direct evidence for an increased oxidative damage with the age of G0 cells, supporting previous conclusions (5). It should be noted also that it has been demonstrated previously that the levels of carbonylation in yeast cells starved for 5 days are lower than in cells starved for 3 month (37). Additionally, we found that, in contrast to the wild-type strain, pos9 mutants were unable to decrease the level of oxidized proteins in the G0 state, whereas yap1 mutants showed a higher level of oxidized proteins only during the exponential growth phase. The Yap1 and Pos9 transcription factors have been demonstrated to control the expression of several common genes, such as *TRX2* and *TRR1* (18), and the redundancy of these systems has been left without any convincing explanation apart from the fact that cadmium resistance seems to be specifically controlled by Yap1p (20). The data presented here, however, indicate that the role of these systems in protecting the cell against self-inflicted oxidation is distinct and intimately linked to different phases of growth of the culture. Specifically, the Pos9 system is involved in protection against oxidative injuries in the G0 phase, whereas the Yap1 system is more important during growth.
Based on our results, we argue that G0 cells of yeast might be an excellent and convenient model system to approaching the free radical hypothesis of aging. Specifically, we were interested in the causal factors behind the apparently universal increase in oxidized macromolecules in aging cells and whether the "rate of living" hypothesis (38) could help explaining this oxidation in G0 cells. It has been observed that there is an apparent correlation between species-specific metabolic rate and life span, such that species with lower metabolic rates consume less O2 per gram of tissue and have a longer maximum life span potential. Moreover, a factor called the "life energy potential," defined as the product of the specific metabolic rate and the maximum life span potential, has been argued to be more or less constant for all species. Therefore, it has been proposed that the rate of energy consumption is a priori responsible for senescence (39), and this concept is referred to as the rate of living hypothesis (38, 40). With the demonstration that respiring mitochondria generate ROS (25), an updated version of the hypothesis states that a faster rate of respiration hastens aging by the greater generation of oxygen radicals. The rate of living hypothesis has, in this form, essentially merged with the free radical hypothesis of aging (9, 41).

When applied to G0 cells, the hypothesis could explain increased oxidation of proteins in resting and nongrowing cells simply due to the fact that such cells have an ongoing respiratory activity but little or no ability to replace damaged proteins. In addition, the prediction of the hypothesis is that the higher the rate of respiration, the greater the oxidation damages and the shorter the life spans. As shown here, our data do not support such a model; no strict correlation between respiratory activity and protein oxidation was found when using different starvation conditions to induce a G0 state. Instead, we demonstrate that a stepwise increase in protein oxidation occurs when the metabolic activity reaches exceedingly low levels. In addition, this elevation of carbonyl levels occurs concomitantly with a respiratory state transition (the RSV drops to 0), and at this time, and subsequently, ATPase inhibitors (e.g. TET) could no longer inhibit respiration. We believe that these results favor the hypothesis that mitochondria and the respiratory apparatus have an increased tendency to generate ROS during growth arrest, which may be linked to a respiratory state transition rather than to the rate of metabolism per se.

The state transition of G0 cells appears to be a consequence of a drastically reduced activity of the oxidative phosphorylation as inferred from results showing that TET failed to reduce respiratory activity as the G0 cells progressed into stationary phase. Moreover, CCCP addition markedly increased respiration of TET-treated cells, demonstrating that the G0 cells were not depleted for reducing equivalents and that the membrane potential was retained. A key question that arises from these observations is why the ATPase appears to be so tightly regulated in starved cells. Since the potential still exists (CCCP stimulates respiration), the most obvious explanation is that the ATP/(ADP + P) ratio is too high for the ATPase to function, a situation that would mimic a classical state transition in experiments with isolated mitochondria. However, the ATP/(ADP + P) ratio is not known, because measurements of the in vivo mitochondrial pools of ATP, ADP, and inorganic phosphate (P) are, as far as we know, technically impossible to perform. Another possibility is a mechanistic change of the ATPase itself that would affect its kinetic properties. This could be due to age-related damage. However, if the ATPase activity is reduced due to a starvation-induced damage, we have to hypothesize that this damage occurs immediately as cells deplete their carbon source (in contrast to nitrogen starved cells), and this seems unlikely in view of the fact that carbon-starved cells recover their respiratory activity immediately when carbon is added back to the culture. Finally, an allosteric change of the ATPase could also change its properties. Present studies are aimed at approaching the possibilities discussed and to understand the primary cause for the state transition observed in G0 cells.

Other questions concern how and why a state transition escalates ROS production and oxidative damage. On isolated mitochondria, a state transition (from state 3 to state 4) occurs when the ADP is depleted, which results in an increase in the membrane potential and a reduced electron flow through the respiratory chain (26). In addition, direct measurements of

![Graph](image-url)

**FIG. 10. Effect of antimycin A and myxothiazol on protein carbonylation and respiration.** Cells were grown in a fed-batch culture similar to the one described in Fig. 4. After ~70 h, three samples of 100 ml each were removed and inoculated into 500-ml stirred E-flasks that were kept at 30 °C. One contained antimycin A, another contained myxothiazol, and the third was used as a control. After 1 h, the rate of respiration and protein carbonyl level was assayed. The levels of carbonylation and respiration were arbitrarily assigned a value of 100 for the untreated control.

**TABLE 1**

| Strains       | Relevant phenotypes                  | Figures in which the strain was used | Origin        |
|---------------|--------------------------------------|-------------------------------------|---------------|
| W303–1A      | MATa; wild type made prototrophic.    | 1, 2, and 4–10                     | Hadi Valadi  |
| BY4741       | MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0 | 3                                   | Euroscarf    |
| BY4741       | MATa; his3Δ1; leu2Δ0; met15Δ0; pos9::kanMX4 | 3                                  | Euroscarf    |
| BY4741       | MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; yap1::kanMX4 | 3                                  | Euroscarf    |
ROS generation on isolated mitochondria from pigeon and rat demonstrated that this reaches maximal levels during a state 4 respiration (25). It has been suggested that the increase in the membrane potential associated with a state 4 respiration is causing an increased generation of ROS (42), and Korshunov et al. (24) have shown a direct relationship between hydrogen peroxide production and the value of the membrane potential. However, the elevated membrane potential is intimately associated with a reduced flow of electrons during a state 4 respiration, and it is difficult to distinguish which of these two factors would have the greatest impact on the generation of ROS. In addition, it has not been established that the results concerning ROS production, membrane potential, and electron flow in isolated mitochondria are applicable to intact cells studied in vivo. The data presented here suggest that a state 4-like respiration affects the degree of oxidative damage in intact yeast cells. Therefore, our results are in line with the conclusions from in vitro experiments.

According to Skulachev’s hypothesis (30), the cytochrome \( b_L \) is highly reduced in state 4, since its oxidation to cytochrome \( b_H \) is prevented by a high membrane potential. In view of the fact that semiquinones (CoQ\(\bullet^-\)) are being oxidized to CoQ by cytochrome \( b_L \), a state 4 could then lead to a long-lived form of semiquinones (CoQ\(\bullet^-\)), which can, by reacting with molecular oxygen, generate superoxide ions (O\(\bullet^-\)). The oxidation observed in intact \( G_0 \) cells was enhanced by treating cells with low concentrations of antimycin A and attenuated with myxothiazol, indicating that protein oxidation is linked to ROS generated by CoQ\(\bullet^-\) of the Q-cycle. These data suggest that the redox status of the quinone pool is directly related to the level of oxidative damage in the cell. This is in line with the suggestion that the CoQ\(\bullet^-\) acts as an intermediate between a high potential and superoxide production. It should be noted, however, that we describe an in vivo pseudo state 4, and this state may not share all the characteristics of the traditional state 4 as defined for isolated mitochondria. Assuming a mechanistic link between electron flow and membrane potential (25), it is likely, however, that a pseudostate 4 is indeed associated with a high membrane potential also in vivo.

In summary, we have established distinctive, growth phase-dependent assignments of the Pos9 and Yap1 transcription factors in protecting the cells against self-inflicted oxidation, and we demonstrate for the first time a link between non-phosphorylating respiration and protein oxidation in vivo and show that the ROS production system responds to the state of respiration regardless of the respiratory rate. We put forward the hypothesis that the redox state of the quinones is the intermediate step between a high membrane potential, ROS production, and oxidative damage to proteins and that the state, rather than the rate of respiration, dictates the degree of self-inflicted damage in growth-arrested yeast cells.

Acknowledgments—We thank Christer Larsson and Manuel Ballesteros for technical and personal help.

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