Oxidative Stress Defense and Deterioration of Growth-arrested Escherichia coli Cells*

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Analysis of protein carbonylation demonstrates that the stasis-induced catalases and cytoplasmic superoxide dismutases (SOD) have a role in preventing accelerated protein oxidation during growth arrest of Escherichia coli cells. A larger number of proteins are carbonylated in cells lacking cytoplasmic SOD compared with cells lacking catalases, OxyR, or RpoS which, in turn, exhibit a larger number of oxidized proteins than the wild-type parent. Proteins exclusively oxidized during stasis in mutants lacking cytoplasmic SOD include GroEL, EF-G, and the acidic isoform of H-NS indicating that these mutants experience problems in peptide elongation and maintaining protein and DNA architecture. These mutants also survive stasis poorly. Likewise, but to a much lesser extent, mutations in oxyR, an oxidative stress regulator, shorten the life-span of stationary phase cells. The low plating efficiency of cells lacking OxyR is the result of their inability to grow on standard culture plates unless plating is performed anaerobically or with high concentration of catalase. In contrast, cells lacking cytoplasmic SOD appear to die prior to plating. Our data points to the importance of oxidative stress defense in stasis survival, and we also demonstrate that the life-span of growth-arrested wild-type E. coli cells can be significantly extended by omitting oxygen.

Growth arrest of Escherichia coli cells caused by starvation for an essential nutrient triggers the production of catalases and other oxidative stress proteins which render the cell highly resistant to hydrogen peroxide, a phenomenon known as starvation-induced cross-protection (1–5). One model, we could call it the future provision model, suggests that this induction of stress defense proteins including oxidative stress proteins prepares the starved cell for stress conditions that may encounter in the future. It has been argued that such a response is sensible at the onset of starvation because energy generation will become more and more limited as cells progress into stationary phase making inducible responses less immediate and less effective (6). Another model suggests that the induction of oxidative stress proteins has a role in minimizing damage to target molecules caused by stasis per se (3, 7, 8). Similarly, it has been proposed that the ubiquitous progressive decline in the functional capacity of aging eukaryotes is a consequence of the accumulation of oxidative damage caused by reactive oxygen species (ROS) produced by normal metabolism (9). This is the postulation of the free radical hypothesis of aging (10). The hypothesis is supported by different experimental data demonstrating that (i) steady-state levels of oxidatively damaged macromolecules increase with age in all species examined thus far (11), (ii) oxidatively modified proteins lose their catalytic activity and structural integrity (12), (iii) there is a close association between oxidative damage of proteins and life expectancy of houseflies (13), and (iv) the life-span of fruitflies can be prolonged by overproducing antioxidants (9). Recent identifications of gerontogenes (genes whose alteration causes life extension) and their functions in both Caenorhabditis elegans (14) and Drosophila melanogaster (15) further support the notion of a strong correlation between longevity and oxidative stress defense.

With the development of sensitive immunochemical methods for the detection of protein carbonyls, the presence of such groups has been used as a marker of ROS-mediated protein oxidation (16) and to demonstrate a correlation between the oxidation of target molecules and aging (17). In this work, we have used such a method to show that the cytoplasmic E. coli superoxide dismutases (SOD), whose levels we demonstrate are elevated upon starvation, and catalases are important in slowing down stasis-induced protein oxidation. By using two-dimensional gel electrophoresis in combination with carbonyl detection, we found that stasis-induced oxidation targets specific proteins and that protein oxidation is both quantitatively and qualitatively different in wild-type cells as compared with cells lacking cytoplasmic SOD and catalases activity and the regulators OxyR and RpoS. Further, viability measurements give support for the hypothesis that oxidative defense proteins prevent oxidative deterioration which, in mutants lacking SOD leads to die-off in the starvation regimen, whereas in OxyR-deficient mutants it leads to a loss in the ability of the cell to recover on standard laboratory nutrient plates. This stasis-induced loss of culturability of oxyR mutants is closely associated to oxidative stress because plating anaerobically or with high levels of catalase increased the plating efficiency by about one order of magnitude. Moreover, we show that the die-off of wild-type E. coli cells during the first 10 days of stasis can be completely counteracted by omitting oxygen, indicating that reactive oxygen species may limit longevity also of wild-type E. coli cells starved under aerobic conditions. However, the life-span of the wild-type stationary phase culture does not appear to depend on protection against oxidative mutations and damage to DNA.

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The abbreviations used are: ROS, reactive oxygen species; SOD, superoxide dismutase; MOPS, 4-morpholinepropanesulfonic acid; ppGpp, guanosine tetraphosphate.
The carbonyl groups in the protein side chains were derivatized, using the Oncor OxyBlot™ kit, to 2,4-dinitrophenylhydrazine (DNP-hydrazone) by reaction with 2,4-dinitrophenylhydrazine (DNP-hydrazone). As described (8), crude protein extracts were obtained during growth at different times in stationary phase, the extracts were run on SDS-PAGE and transferred to polyvinylidene difluoride membrane, and oxidatively modified proteins were detected with anti-DNP antibodies.

**RESULTS**

The Rates of SodA and SodB Production Increase upon Starvation of Cells—Cells of _E. coli_ were pulse-labeled with [3H]leucine during exponential growth and also at times after growth ceased because of glucose depletion. Analysis by two-dimensional gel electrophoresis revealed that the rates of synthesis of both SodA (Mn-superoxide dismutase) and SodB (Fe-superoxide dismutase) were induced during the starvation condition studied. The identifications of SodA and SodB in the two-dimensional gene-protein data base (23) was verified by N-terminal sequencing. The location of these proteins on the _E. coli_ reference two-dimensional gels (SodB on isoelectric focusing gel and SodA on nonequilibrium pH gel electrophoresis gel) and the extent and kinetics of induction during carbon starvation are depicted in Fig. 1, A and B. As seen in Fig. 1B, induction of SodA synthesis preceded SodB. A _sodA::lacZ_ fusion demonstrated that the increased synthesis of SodA was the result of transcriptional activation during stasis (not shown). The increased rate of SodA and SodB synthesis during stasis does not appear to be an effect of a gradual reduction in growth rate upon entry into stationary phase because the production rates were found to be indistinguishable at steady state growth rates between _k_ = 0.3 to 1.9 (Fig. 1C). For a comparison, the rate of SdhA (succinate dehydrogenase flavoprotein subunit) production, known to be inversely dependent on growth rate (24), is also depicted in Fig. 1C.

These results, together with the fact that growth-arrested cells accumulate catalasase (25), indicate that the capacity of the cell to deal with ROS generated from endogenous metabolism increase during stasis. The question of whether these enzymes indeed are needed to prevent oxidation of macromolecules during growth arrest was approached by determining the levels of protein carbonyls during stasis of wild-type cells and cells lacking SOD and/or catalasase activities.

**Stasis-induced Protein Carbonylation Is Elevated in Cells Lacking Superoxide Dismutase and Catalase Activity**—The carbonyl content of total proteins was measured immunochemically, and densitometric quantification of the blots demonstrated a 4- to 5-fold increase in carbonyl content in wild-type cells during a period of 2 days in stationary phase (Fig. 2) as previously demonstrated (8). Mutants lacking cytoplasmic SOD activity (sodA sodB double mutants), catalasase activity (katE katG double mutants), or both exhibited enhanced (about 10-fold) carbonyl content during stasis (Fig. 2). No, or only minor, differences in protein carbonyl levels were observed between wild-type and mutant cells growing exponentially (Fig. 2). Protein carbonylation could not be detected when cells were grown and growth-arrested anaerobically (not shown).

**Stasis-induced Carbonylation Targets Specific Proteins**—Two-dimensional gel electrophoresis in combination with immunchemical assay for protein carbonyl groups (26) has demonstrated that some proteins are specifically susceptible to stasis-induced oxidation (8). In Fig. 3, we demonstrate that a larger fraction of proteins are oxidized in all oxidative defense mutants analyzed as compared with the wild-type strain and that the largest number of oxidized proteins is present in the mutants lacking cytoplasmic SOD activity. No significant difference between wild-type and mutant strains were detected during exponential growth of cells (only one, unidentified, protein was found to be significantly oxidized during exponential growth in LB; not shown). By overexposing the films, we were able to determine whether some proteins were exclusively oxidized in response to stasis in cells lacking cytoplasmic SOD activity. We found that the heat shock chaperone GroEL, elongation factor EF-G, carbamoylphosphate synthetase (small subunit, CarA), pyruvate formate lyase (Pfl), and the acidic isoform of H-NS were oxidized only in cells lacking cytoplasmic SOD and SOD/catalasase activity (Fig. 4). This result cannot be explained by an increased level of these proteins in the mutant strain because Coomassie Brilliant Blue staining of proteins demonstrated that the levels were very similar in the wild-type and mutant backgrounds (not shown). One exception, GroEL,
The Rate of Stasis-induced Die-off Is Increased in Mutants Impaired in Oxidative Stress Defense—As shown in Table II, sodA sodB mutants were found to be markedly impaired in their ability to survive stasis, whereas katE katG double mutants were only modestly, but reproducibly, impaired in stasis survival. The superoxide dismutase and catalase activities appear to synergistically protect stationary phase cells as judged by the poor survival of the sodA sodB katE katG quadruple mutant (Table II). It should be noted that stationary phase death of a sodA sodB double mutant has been demonstrated previously in another genetic E. coli background (27).

The OxyR (28) and RpoS (29) regulons are known to be involved in oxidative stress defense, and rpoS mutants are known to survive stationary phase poorly (30). In addition, we have previously shown that the total protein carbonyl levels are enhanced in both oxyR and rpoS mutants during stasis (8). We found that oxyR mutants, like rpoS mutants (30), are impaired in stationary phase survival (Table II). In addition, the yield of oxyR colony-forming units/ml was always about 10-fold lower than that of the wild-type culture in early stationary phase.

We next asked whether the fraction of cells that failed to form colonies on the LB plates in fact died in the stationary phase regimen or were killed upon plating. We approached this question by comparing plating efficiencies on standard LB plates incubated anaerobically and aerobically with and without catalase (5,000 units), arguing that the increased stasis-induced protein oxidation observed in the different mutants (Ref. 8, and this study) may degenerate the cells to the extent that they fail to cope with the subsequent burst of aerobic catabolism when plated on a rich growth medium. As seen in
Table II, both anaerobiosis and catalase addition increased the plating efficiency of oxyR and katE katG mutants to levels comparable with the wild-type strain. However, anaerobiosis and addition of catalase did not increase the plating efficiency of wild-type cells or sodA sodB double mutants (Table II).

Similarly, anaerobiosis and catalase additions did not increase the plating efficiency of stationary phase rpoS mutants (not shown).

It should be noted that protein oxidation preceded stationary phase die-off of mutant cultures indicating that death per se is not the causal factor responsible for increased carbonylation. The Surviving Fraction of a Stationary Phase Culture Does Not Depend on Protection Against Oxidative Mutations and Damage to DNA—It is possible that stasis sensitivity of cells lacking oxidative defense proteins is caused by increased DNA damage and mutation frequencies. Indeed, the sodA sodB double mutant was found to have about two-fold higher mutation frequencies than the wild-type in stationary phase (measured by the occurrence of rifampicin-resistance mutants). However, we think that this increase in mutation frequency is of no consequence for the survival of the cells because stationary phase survival of a mutT mutant, which has a 300 to 1,000-fold increase in mutation frequency during stasis, was found to be indistinguishable from the wild-type parent (Table II). We also determined stasis survival of DrecA and DrecA dps double mutants which are unable to cope with oxidative DNA damage. Again, we found no evidence that oxidative damage to DNA limits survival during stasis because the colony forming capacity of the wild-type and DrecA or DrecA dps double mutant strains was very similar during the first 3 days in stationary phase (Table II).

The Life-span of a Growth-arrested Wild-type Culture Is Increased by Omitting Oxygen—The data presented suggest that endogenous generation of ROS creates a serious problem in mutants lacking cytoplasmic SOD and SOD/catalases activity during conditions of growth arrest (Table II). To investigate whether the longevity also of growth-arrested wild-type E. coli cells is limited by self-inflicted oxidative damage, we deter-
**Stasis-induced Radical Scavenging in E. coli**

FIG. 5. The life-span of wild-type *E. coli* cultures starved for glucose aerobically (■) or anaerobically (■). Representative results are presented in the figure and the standard deviation was always less than 10%.

mined and compared the life-span of cultures starved for carbon aerobically and anaerobically. The cells were grown aerobically rather than anaerobically prior to starvation to avoid production and excretion of mixed acid fermentation products which are potentially toxic and can seriously debilitate the membrane. As depicted in Fig. 5, the culture life-span of anaerobically starved cells significantly exceeded that of the aerobically starved counterpart. About 98% of the culture which was glucose-starved aerobically died within 10 days of starvation whereas no significant killing of cells in the anaerobic culture was observed during the same period.

**DISCUSSION**

Starvation-induced growth arrest results in protein oxidation in wild-type cells of *E. coli* and is enhanced in cells lacking cytoplasmic SOD and/or catalases activity. Striking differences in total carbonyl levels were observed between the mutant and wild-type cells only in stationary phase, which suggests that these functions are especially important during this period. We believe that this self-inflicted gradual increase in protein oxidation during stasis is caused by an imbalance between macromolecular synthesis and endogenous catabolism. The rate of macromolecular synthesis is drastically reduced or totally blocked upon starvation because of the lack of precursor metabolites and the control by the alarmone ppGpp (31). However, the rates of respiration (22) and production of reactive oxygen species (32) are not reduced to the same extent and proceed at relatively high levels for an extended period during stasis. Thus, the growth-arrested cell, in contrast to the exponentially growing one, has only limited abilities to titrate out time-dependent oxidation of target macromolecules and the levels of such damaged molecules will increase unless the oxidative defense machinery can fully repair or remove them. It appears that a significant number of the genes and regulons induced by stasis is indeed part of such a defense machinery which, however, fails to fully combat stasis-induced oxidation.

The two-dimensional carbonyl immunoassay demonstrates that stasis-induced protein oxidation (carbonyls groups could be introduced at lysine, arginine, proline, and threonine residues (17)) is selective. Based on the identity of the oxidized proteins, we can conclude that several different cell processes are targets for stasis-induced damage. In the wild-type strain, these functions include peptide chain elongation (EF-Tu), protein folding and reconstruction (DnaK), DNA architecture and gene expression (H-NS, basic isoform), central carbon catabolism (Icd, Mdh, AceF, SucC, Pyk, PtsI), amino acid biosynthesis and nitrogen assimilation (GlnA, GltD), and general stress protection (UspA) (8). Additional proteins, including GroEL, EF-G, and the acidic isoform of H-NS, were found to be oxidatively damaged in the sodA sodB and the sodA sodB katE katG strains, indicating that these strains experience even larger problems in maintaining translation, proper protein folding, and DNA architecture during stasis. We do not know whether the specific sensitivity of some proteins to oxidation is the result of design rather than necessity or chance. For example, it is possible that metal-catalyzed oxidation will be an intrinsic problem for all proteins containing, or being associated with, metals like e.g. iron and manganese (16, 33). It is known that a number of different ROS are involved during the course of the protein oxidation process and that transition metal ions can substitute for hydroxyl groups and superoxide radicals in some of the reactions (17).

Two possible models explaining the role and causation for stasis-induced stress protein production have been discussed in the literature. One model suggests that the induction of stress defense proteins and regulons prepares the growth-arrested starved cell for future cataclysmic stress conditions (future provision model). Another model suggests that the induction of stress defense regulons during stationary phase has a role in minimizing damage to target molecules caused by stasis per se (3, 7). In the latter model, it is argued that stasis causes increased damage (e.g. by oxidation) of cellular components, and stress resistance develops because the cells are already exposed to the normal stress response signals. Support of this model, with respect to induction of heat shock genes during stasis, was recently presented by demonstrating that aberrant proteins are the likely candidates triggering induction of heat shock regulon during both heat stress and stasis but the signal (aberrant proteins) is generated by different pathways (8). The same argument can be made for stasis-induced expression of OxyR-dependent genes. OxyR becomes an active regulator upon oxidative formation of a disulfide bond between two of its cysteine residues (34), and it is feasible that the critical cysteine residues on OxyR are subjected to gradual oxidation during stasis, which eventually may give rise to a high enough titer of oxidized OxyR to activate gene expression (Indeed, formation of disulfide bonds in a cytoplasmic alkaline phosphatase has recently been shown to occur during stasis of *E. coli* cells (8)). Moreover, the data concerning the role of superoxide dismutases, catalases, OxyR, and RpoS in preventing stasis-induced protein oxidation and promoting stasis survival (this work; Refs. 7 and 27) suggests that many stress proteins are not just for future provision but are actively participating in stationary phase physiology. However, it should be noted that the two models are not mutually exclusive. It is possible that oxidative stress proteins have a dual role of enhancing the chances of survival of the growth-arrested cell by damage repair and protection and at the same time provide for the future. It may be difficult to explain the origin and evolution of the genetic program directing stasis induction of oxidative stress systems by the future provision model unless we propose that starved cells almost always will encounter future oxidative stress. Perhaps the process of recovery and subsequent regrowth of stationary phase cells subjected to up-shift conditions is intimately associated with such oxidative stress. For example, a sudden burst in catabolic activities preceding macromolecular biosynthesis during recovery could generate severe damage unless the cells are already provided with a battery of
oxidative defense systems. The data presented in Table I lend some credence to this notion by demonstrating that the poor plating efficiency of stationary phase katE katG and oxyR cells can be counteracted by anaerobiosis or including catalase in the LB plates. Thus, stasis induction of oxidative stress systems could perhaps be explained by both models, and it appears as if superoxide dismutases are important in protecting the cell against primary damage caused by ongoing metabolism during stasis per se while catalases and the OxyR regulon may be required during secondary oxidative stresses associated with recovery during upshift conditions. However, it is important to note that the failure of oxyR and katE katG mutants to grow when plated aerobically only occurs after the cell have been subjected to growth arrest for some time, indicating the involvement of a progressive stasis-dependent deterioration.

Based on the results demonstrating stasis-induced oxidation of target proteins and the poor ability of cells lacking components of oxidative stress systems to survive (or recover from) stasis, it may seem reasonable to propose that ROS and oxidative stress is a major cause of stationary phase death. This proposition would be in accord with the free radical hypothesis of aging. However, this, and most contemporary papers concerning stationary phase survival in bacteria, concerns the analysis of mutants that are worse off than the wild-type strain, and we cannot make the a priori assumption that wild-type E. coli dies for the same reason as those mutants. It is clear, however, that the life-span of growth-arrested wild-type E. coli cells can be significantly extended by omitting oxygen and that a significant number of genes induced by stasis are intimately associated with the protection against endogenously generated oxidative damage.

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