Protein oxidation in response to increased transcriptional or translational errors

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In this study, we show a correlation between synthesis of aberrant proteins and their oxidative modification. The level of aberrant proteins was elevated in *Escherichia coli* cultures by decreasing transcriptional or translational fidelity using specific mutations or drugs. Protein carbonylation, an oxidative modification, increased in parallel to the induction of the heat shock chaperone GroEL. As the protein turnover rates and level of intracellular oxidative stress remained unchanged, it appears that carbonylation results from the increased susceptibility of the misfolded proteins. These studies show that the cellular protein oxidation is not limited only by available reactive oxygen species, but by the levels of aberrant proteins. Thus, protein oxidation seen in aging cells may be the consequence also of reduced transcriptional/translational fidelity, and protein structures appear to have evolved to minimize oxidative damage. In addition, we discuss the possibility that carbonylation, being an unrepairable protein modification, may serve as a tagging system to shunt misfolded proteins between pathways of refolding by chaperones or the proteolytic apparatus.

The free radical hypothesis of aging postulates that the ubiquitous progressive decline in the functional capacity of aging cells or organisms is a consequence of the accumulation of oxidative damage caused by reactive oxygen species (ROS) produced by normal metabolism (1, 2). The hypothesis is supported by experimental data demonstrating that the steady-state levels of oxidatively damaged macromolecules increase with age and that the life span of some organisms can be prolonged by overproducing antioxidants (1–4). The increased levels of oxidized proteins is often associated with an age-related diminished capacity of the oxidative defense systems (5, 6). However, it is also possible that increased protein oxidation is a result of increased susceptibility to ROS due to subtle alterations in the structure of abnormal proteins. For example, it is possible that mistranslated or otherwise misfolded proteins become more susceptible to oxidation and, if aberrant proteins accumulate during aging, so will the oxidized proteins.

The study of the mortality of bacteria and how they survive starvation-induced growth arrest has raised the question of whether the free radical hypothesis of aging is relevant also for explaining the progressive deterioration of growth arrested bacterial cells. *Escherichia coli* cells, like cells of multicellular organisms, also show an age-related increase in protein oxidation, and recent data directly support the idea that stasis-induced deterioration of proteins may be a problem in growth-arrested bacteria (7). Based on the identity of oxidatively modified proteins, it has been suggested that aging *E. coli* cells may experience problems in performing peptide chain elongation, protein folding and reconstruction, central carbon catabolism, and nitrogen assimilation (7).

In this study, we have used *E. coli* as a model organism to test whether aberrant proteins are more susceptible to oxidation. We demonstrate that increasing the production of aberrant proteins in a number of different ways results in an increase in the levels of oxidized proteins (carbonylation) despite an increased turnover rate. Thus, aging cells may accumulate aberrant misfolded proteins which then become oxidized, rather than the other way around.

Materials and Methods

Chemicals and Reagents. Detection of carbonylated proteins was performed using the chemical and immunological reagents of the OxyBlot oxidized protein detection kit (Oncor). Anti-GroEL monoclonal mouse antibodies were purchased from StressGen Biotechnologies (Victoria, Canada). Anti-mouse IgG peroxidase conjugates were from Sigma. The chemiluminescence blotting substrate (POD) was obtained from Roche Molecular Biochemicals and used according to instructions provided by the manufacturer. Immobilon-P polyvinylidene difluoride membrane was obtained from Millipore. Protein assay reagents were purchased from Pierce. X-Omat AR-5 was purchased from Eastman Kodak. The ampholines (Resolyte 4–8) used for two-dimensional electrophoresis were obtained from BDH.

Bacterial Strains, Plasmid, and Media. All strains are *E. coli* K-12 derivatives. Mutations in various genes were introduced by P1 transduction in the same parental strain, MG1655 (8), using P1 vir and selecting for an antibiotic resistance marker associated with the mutation (9, 10). The Δ*mutT* (10) strain was generated by cotransduction of the Δ*mutT* allele with *leu*::*Tn10*. pKK726G and pKK3535 (11) were introduced by transformation into the same parental strain MG1655 and selected for carbenicillin resistance. Cultures were grown aerobically in liquid Luria-Bertani medium in Erlenmeyer flasks in a rotary shaker. When appropriate, the medium was supplemented with kanamycin (50 μg/ml), carbenicillin (200 μg/ml), and/or tetracycline (20 μg/ml).

General Methods. P1 transductions and plasmid transformations were performed as described by Miller (12) and Sambrook *et al.* (13). Crude cell extracts were obtained using an SLM–Aminco French Pressure Cell (SLM–Aminco, Urbana, IL). Culture samples were processed to produce extracts for resolution on two-dimensional polyacrylamide gels according to the methods of O'Farrell (14) with modifications (15).

Challenge Conditions. *E. coli* overnight cultures were diluted 100-fold in Luria-Bertani with the addition of various concentrations of streptomycin or puromycin, and cells were allowed to grow until the cell density reached an $A_{600} = 0.5 \pm 0.05$. Crude

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Abbreviation: ROS, reactive oxygen species.

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cell extracts were obtained and processed for carbonylation assays.

Carbonylation Assays. The carbonyl groups in the protein side chains were derivatized, using the Oncor OxyBlot kit, to 2,4-dinitrophenylhydrazone by reaction with 2,4-dinitrophenylhydrazine. As described (7), crude protein extracts were obtained during growth, the proteins reacted with carbonyl reagent, 2,4-dinitrophenylhydrazine, dot-blotted onto polyvinylidene difluoride membranes or separated by two-dimensional electrophoresis, and oxidatively modified proteins were detected with anti-2,4-dinitrophenyl hydrazone antibodies. To determine the intracellular stability of bulk and carbonylated proteins, we used the technique described previously (16).

Antioxidant Measurements. Superoxide dismutase was assayed using the xantine oxidase/cytochrome *c* method (17). One unit of superoxide dismutase is defined as that amount of enzyme which inhibits the rate of cytochrome *c* reduction by 50% at 25°C. Catalase activity in bacterial extracts was determined by measuring the decrease in the A_{240} of hydrogen peroxide as described previously (18). One unit of catalase is defined as that amount of enzyme which decomposes 1 μmol of hydrogen peroxide in 1 min at 25°C.

Superoxide Anion Production. The rate of superoxide generation during *in vitro* reactions was measured as the superoxide dismutase-sensitive rate of cytochrome *c* reduction. Reduction of cytochrome *c* was monitored spectrophotometrically at 550 nm ($E = 24\ m^{-1}\ Mcm^{-1}$) in a 1-ml reaction mixture consisting of 50 mM phosphate buffer (pH 7.4), 20 μM cytochrome *c*, and 100 μM NADH with and without 50 units of superoxide dismutase. Production of superoxide anion was measured in isolated respiratory vesicles and cytosolic fractions prepared as previously described by Imlay and Fridovich (17).

Results

Streptomycin Treatment Induces Protein Carbonylation. In view of the fact that both oxidized and aberrant proteins accumulate in aging cells, we tested whether aberrant proteins themselves exhibit an increased susceptibility for oxidation. Protein oxidation was followed by measuring protein carbonylation which is an irreparable protein modification (19) detectable by an immunochemical method. Altered proteins were produced by treatment of cells with streptomycin, an antibiotic well known to increase mistranslation (20) and a concomitant induction of heat shock chaperones (15). Streptomycin was added to growing cells at concentrations low enough not to affect the rate of cell growth, and the carbonyl content of total protein was measured. The levels of protein carbonylation increased up to 9-fold at 1 μg/ml streptomycin, demonstrating that mistranslation increases protein oxidation (Fig. 1). We also tested whether the level of misfolded protein induced by streptomycin treatment was sensed by the cells through an increased expression of chaperones. GroEL (the hsp60 homologue of *E. coli*) levels increased concomitantly with, and proportionally to, protein carbonylation, suggesting an association between misfolding and carbonylation (Fig. 1). The concentration of streptomycin used did not cause an increased production of superoxide or primary and secondary oxidative stress proteins, including SodA, KatE, Dps, and GorA (Fig. 1), nor did it result in an increased oxidation of cysteine residues (disulfide bonds) in a cytoplasmic alkaline phosphatase (Δ2–22AP; ref. 7) or reduced superoxide dismutase and catalase activities (Fig. 1), demonstrating that streptomycin did not cause a general oxidative stress.

We further tested whether protein carbonylation was specific for just a few proteins or was a general phenomenon. Two-dimensional gel electrophoresis followed by detection of carbonyl groups demonstrated that most, if not all, protein species detected were carbonylated and that protein "stuttering" (i.e., mistranslation generating several isoforms of the same protein) can be observed (Fig. 2). These results demonstrate that most mistranslated proteins generated by streptomycin treatment are susceptible to protein carbonylation. This is in contrast to carbonylation during oxidative stress where only a few specific proteins are oxidatively modified (Fig. 2; ref. 7).

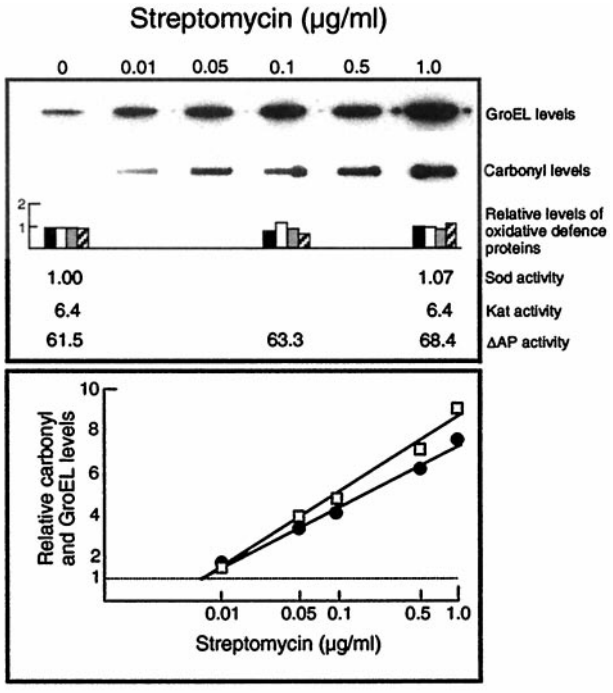

Fig. 1. Effects of streptomycin on total carbonyl content (□) and GroEL production (●) in *E. coli* MG1655. Cells were treated with streptomycin concentrations ranging from 0.01 to 1 μg/ml, which have no effect on growth rate (data not shown), and crude extracts were used for carbonyl content quantification (7). In the same samples, GroEL levels were determined by Western blot analysis using monoclonal mouse anti-GroEL antibodies. Quantification of carbonyl and GroEL levels were obtained using the ImageQuant software (Molecular Dynamics). The carbonyl and GroEL levels in the nontreated controls were assigned a value of 1.0. The relative rates of SodA (black bars), KatE (open bars), Dps (gray bars), and GorA (hatched bars) production in nontreated and streptomycin-treated cells were determined as described (7). Sod and Kat activity was determined and expressed as described in *Materials and Methods* and the extent of disulfide bond formation (ΔAP activity) was as described in refs. 7 and 22. The analysis was repeated three times to confirm reproducibility. Representative results are presented and the SD was always <20% between experiments.

Correlation Between Mistranslation and Protein Carbonylation. To test the generality of the results obtained with streptomycin, we used three other means of producing aberrant proteins: (*i*) addition of puromycin which causes premature translation termination, resulting in truncated polypeptides (21); (*ii*) introduction of the multicopy plasmid pKK726G which carries a mutated gene for 16S rRNA [ribosomes incorporating the mutant rRNA are prone to mistranslation (11)]; and (iii) introduction of a mutation in *mutT* [*mutT* mutants exhibit decreased transcriptional fidelity causing mistranslation via mistranscription (10)]. All of these different means of increasing error rates in protein biosynthesis caused an increase in total protein carbonylation (Fig. 3 and 4), an increase in GroEL levels, and, in the one case analyzed (*mutT* mutants), most proteins appear to be carbonylated as demonstrated by two-dimensional gel electrophoresis

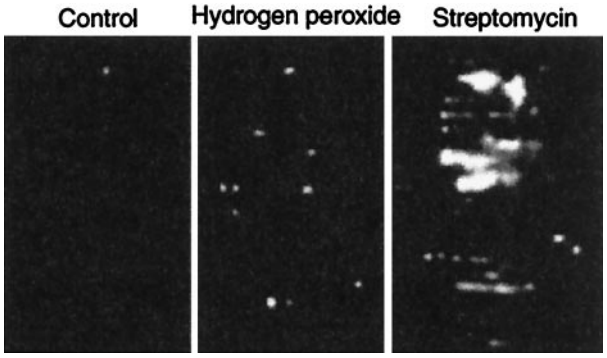

Fig. 2. Protein carbonylation determined by two-dimensional Western blot immunoassay. Crude extracts from cells treated with 0 and 1 μg/ml of streptomycin and 200 μM hydrogen peroxide were processed for resolution on two-dimensional polyacrylamide (19) with modifications (20). Autoradiograms were obtained after carbonyl immunoassay of proteins (5). The analysis was repeated three times to confirm reproducibility. Representative results are shown.

(data not shown). Measurements showed that the rate of superoxide production and the activity of the superoxide dismutases and catalases are unchanged in the *mutT* mutant compared with its isogenic parent strain (Fig. 4). In addition, the levels of cytoplasmic disulfide bridge formation (ref. 22; data not shown) and the expression of oxidative defense genes, such as *dps* (ref. 23; Fig. 4) did not increase as a result of inactivating *mutT*, demonstrating that this strain, like streptomycin-treated cells (Fig. 1), does not exhibit increased oxidative stress. These results suggest that cellular protein oxidation during exponential growth of *E. coli* is not limited by available ROS, but by the levels of aberrant proteins.

Carbonylated Proteins Are Rapidly Degraded *In Vivo.* The results described could, as argued above, be explained by an increased susceptibility of mistranslated and/or truncated proteins to carbonylation. However, based on the data presented, we cannot rule out the possibility that the increased protein oxidation is due, in part, to a decreased turnover of oxidized proteins, e.g., because of titration of the proteases by the excess of aberrant proteins in streptomycin-treated cells. To approach this, we

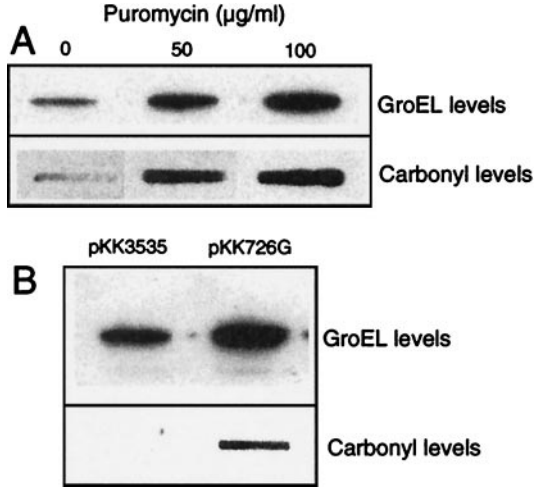

Fig. 3. Correlation between mistranslation and protein carbonylation. Two methods to induce mistranslation were tested: (*A*) addition of various puromycin concentrations and (*B*) transformation of cells with pKK726G which contains a single base change in 16S rRNA (C-726 to G) and a control plasmid, pKK3535, which contains the complete *E. coli* rrnB operon. Because MG1655 carrying pKK726G has a temperature-sensitive phenotype, cells were grown at 30°C. Quantification of carbonyl content and GroEL levels was performed as described in the legend to Fig. 1. The analysis was repeated three times to confirm reproducibility. Representative results are shown and SD was always $<$20%.

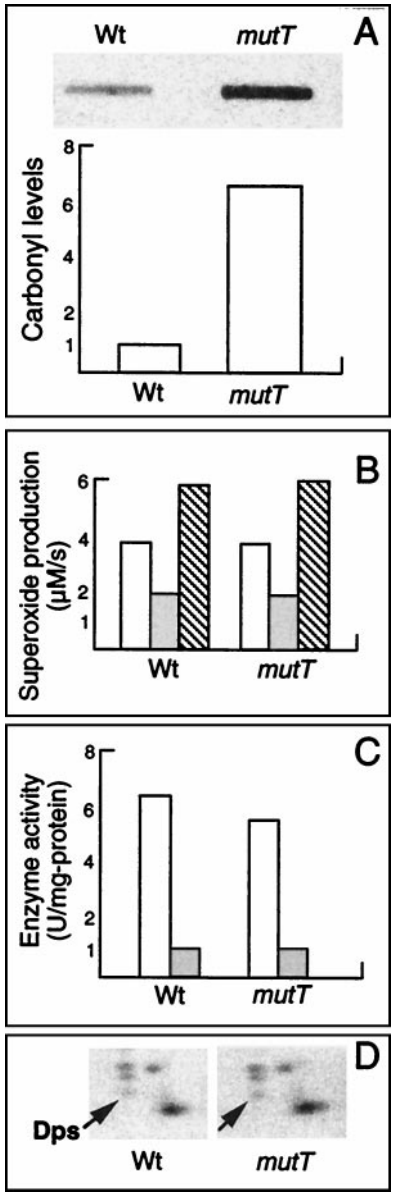

Fig. 4. Carbonylation, superoxide production, superoxide dismutase and catalase activity, and oxidative stress protein production in the *mutT* mutant compared with the wild-type strain. (*A*) Protein carbonyl levels in the wild-type (MG1655) and the *mutT* mutant strain. (*B*) Production of superoxide in the membrane vesicles (open bars), cytosolic fraction (gray bars), and total protein fraction (hatched bars) of a *Φ(sodA′-lacZ)49 Φ(sodB-kan)Δ2* and a *Φ(sodA′-lacZ)49 Φ(sodB-kan)Δ2 ΔmutT leu::Tn10* strain. Each value represents the mean of triplicate determinations at two different protein concentrations and the variation between the measurements was $<$5%. (*C*) Catalase (open bars) and superoxide dismutase (gray bars) activity in crude extracts obtained from wild-type (Wt) and *mutT* mutants. Each value represents the mean of triplicate determinations at two different proteins concentration and the variation between the measurements was $<$10%. (*D*) Rate of synthesis of the oxidative stress protein Dps in the wild-type and *mutT* mutants. The autoradiograms show part of the two-dimensional gel with the Dps protein indicated with the arrow. The cells were pulse labeled during exponential growth (A_{420} = 0.4 $\pm$ 0.05) with radioactive methionine as described previously (15).

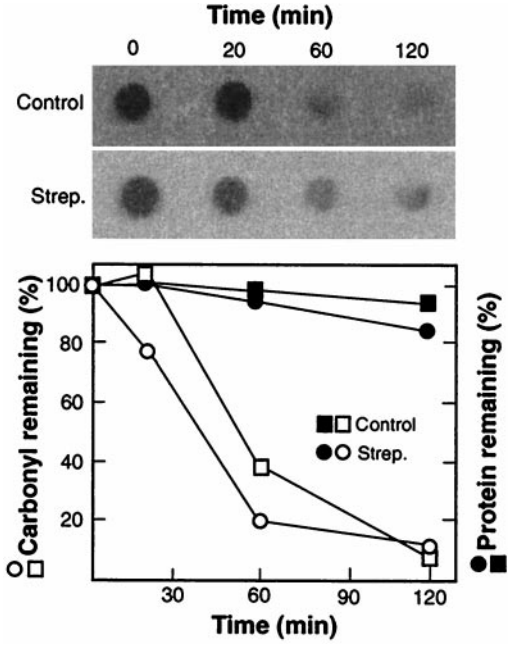

Fig. 5. Turnover of bulk (●, ■) and carbonylated (○, □) proteins in streptomycin-treated (○, ●) and nontreated (□, ■) control cultures. The relative stability of total protein or carbonylated proteins was determined after protein synthesis was inhibited by spectinomycin (100 μg/ml) as described in ref. 16. Spectinomycin was added at an OD_{600} of 0.5. Mistranslation was induced by adding 1 μg/ml streptomycin to the cultures several generations before the block in protein synthesis. Quantification of carbonyl content was performed as described in the legend to Fig. 1.

measured the rate of degradation of carbonylated proteins in streptomycin-treated and nontreated control cultures. We found, first, that carbonylated proteins are degraded much more rapidly than total bulk proteins (Fig. 5) and, second, that degradation is indistinguishable in streptomycin-treated and nontreated cells. Thus, we conclude that the increased oxidation observed in streptomycin-treated cells cannot be the result of a slower degradation of carbonylated proteins in cells exposed to this drug.

Discussion

The data presented here demonstrates, for the first time, that mistranslated proteins appear to be more susceptible to protein oxidation and we conclude that cellular protein oxidation is not limited by available ROS, but by the levels of aberrant proteins. This notion opens up new ways to approach the causal factors behind protein oxidation in aging cells. The increase in protein oxidation observed in aging *E. coli* cells (7), and perhaps other organisms as well, may not always be due to a decreased ability to combat oxidation but by an increase in the substrate for oxidation, i.e., aberrant proteins. At least one kind of mistranslation, frameshifting, is known to increase in *E. coli* cells when they are starved for an essential nutrient (24), and it is possible that this form of mistranslation could account for part of the protein oxidation observed in growth-arrested deteriorating cells of *E. coli* (7). In addition, an obvious interpretation with respect to the susceptibility of aberrant proteins to oxidation is that the native protein structure/folding has evolved to minimize oxidative damage to proteins in an environment of high oxidative potential.

Our results also provide a new platform in approaching the largely unsolved problem of the nature of the signals that target proteins for proteolysis and how the cell decides between the pathways of refolding and degradation for any given substrate. It has been shown that oxidatively damaged proteins are more susceptible to proteolysis than their normal counterparts and it has been suggested that carbonylation, being an irreversible modification, renders the modified protein destined for degradation in oxidatively stress cells (25–32). Indeed, we found that carbonylated proteins are degraded much more rapidly than bulk proteins *in vivo* and carbonylation following mistranslation could signal that a protein is irreparable (by chaperones, for instance) and serve as a tag for the proteolytic pathway. Thus, it is possible that carbonylation tagging of aberrant proteins serves to keep the macromolecular synthesis machinery of the cell free from mistranslated proteins which may otherwise reduce the efficiency or accuracy of information transfer from DNA to protein.

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