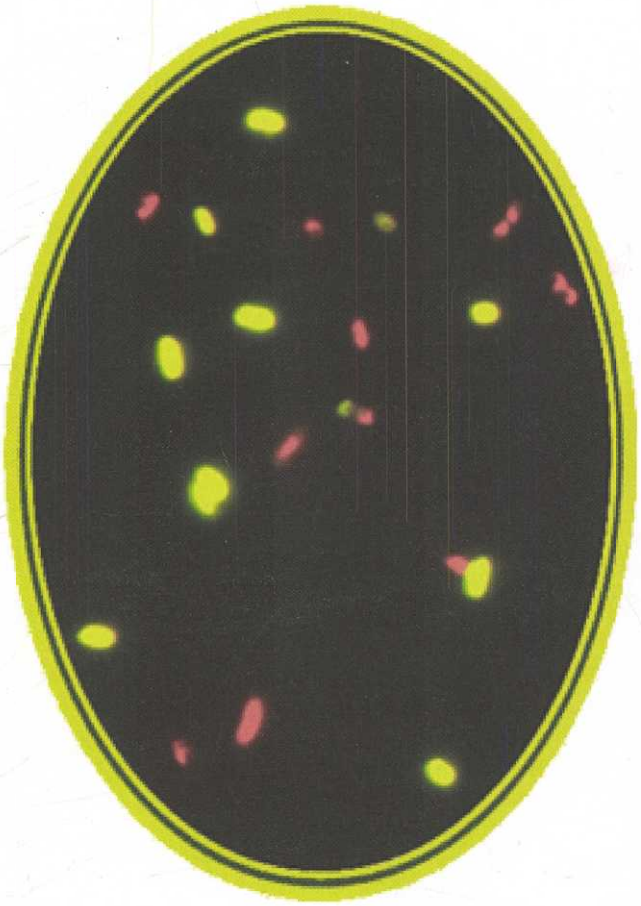
On the Role of Protein Oxidation and Heat Shock Proteins in Senescence and Fitness



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Akademisk avhandling

för filosofie doktorsexamen i mikrobiologi (examinator Professor Thomas Nyström), som enligt fakultetsnämndens beslut kommer att offentligt försvaras fredagen den 12 maj 2006, kl. 10.00 i föreläsningssal Åke Göransson, Medicinaregatan 9, Göteborg

This thesis is based upon the following papers which are referred to by their roman numerals:

I Ballesteros, M., **Fredriksson, Å.**, Henriksson, J., and Nyström, T. 2001. Bacterial senescence: protein oxidation in non-proliferating cells is dictated by the accuracy of the ribosomes. *EMBO Journal* 20: 5280–5289

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Abstract

Similar to the ageing process of eukaryotes, oxidative damage to cellular macromolecules may be involved in deterioration of growth arrested (stationary phase) *Escherichia coli* cells; a process referred to as 'conditional cell senescence'.

In this work we demonstrate that the heat shock proteins (Hsps) are key players in the cellular defence against deleterious protein oxidation (carbonylation) during conditional senescence in *E. coli* cells, that such oxidation is linked to increased production of aberrant proteins caused by increased mistranslation, and that carbonylation of aberrant proteins, which are intrinsically sensitive to oxidation, can occur in the absence of increased oxidative stress. Hsp70 (DnaK), together with the Lon and ClpQY Hsp proteases, are shown to be major participants in protecting stationary phase cells against accumulation of carbonylated proteins. A further link between protein oxidation and Hsps were established by results showing that induction of the heat shock regulon in response to increased mistranslation requires oxidative modification of the malformed proteins. This is shown to be true both for cells entering stationary phase and for cells in which the ribosomes display reduced translational fidelity due to mutations in the ribosomal accuracy centre. In addition to affecting Hsp regulation, mistranslated and oxidized proteins, also affect stationary phase elevation of the transcription factor, SigmaS (σ^S) and induction of the σ^S regulon. Mechanistically, this effect of mistranslation on σ^S acts via titration of the ClpP-protease (ClpXP is responsible for σ^S degradation). σ^S is a key player in switching gene expression from growth/reproduction related activities towards those of maintenance and is essential, similar to the Hsps, to counteract protein oxidation upon entry of cells into stationary phase.

Furthermore, using *Salmonella enterica* serovar Typhimurium LT2 we demonstrate that random mutations achieved during evolution interact such that their combined effect on fitness is mitigated (antagonistic epistasis). The levels of DnaK and GroEL were elevated in lineages with many point mutations. Also, ectopic overproduction of GroEL was demonstrated to increase fitness in such strains. These data suggest that chaperones may buffer the cell against the fitness cost caused by the accumulated mutations and provides a mechanistic, physiological, explanation for antagonistic epistasis.

Keywords: *Escherichia coli*, senescence, fitness, protein oxidation, protein carbonylation, heat shock proteins, Hsp70, DnaK, GroEL, Lon, ClpXP, proteolysis, Sigma32, SigmaS, antagonistic epistasis, *Salmonella*

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Aim of this study and the findings in brief

Cells of the prokaryote model organism, *Escherichia coli*, (*E. coli*) exhibit an elevated oxidation of proteins during growth arrest; a phenomenon that has been suggested to trigger the deteriorative process in these cells that has been referred to as 'conditional cell senescence'. The aim of this work was to search for systems involved in counteracting and protecting the cell against such oxidation and to elucidate to what extent protein oxidation triggers the induction of the defence systems during conditional senescence. The results generated may hopefully inspire researchers interested in the senescence of mandatory aging organisms to look for similar pathways and phenomenon.

The data obtained in approaching the questions and aims has been summarized in 5 papers and the findings included in these papers are briefly outlined below:

(PAPER I (10)): Protein oxidation (carbonylation) in growth arrested cells is shown to occur in the absence of increased oxidative stress. Instead, it is demonstrated that elevated protein carbonylation is a result of increased mistranslation and consequentially increased production of aberrant proteins, which are sensitive targets of carbonylation. This carbonylation of aberrant proteins leads to increased production of heat shock proteins (Hsps), such as the chaperones Hsp70 (DnaK) and Hsp60 (GroEL).

(PAPER II (53)): This paper demonstrates that the accumulation of carbonylated proteins in growth arrested cells can be counteracted by overproduction of the Hsps. DnaK, together with the Lon and ClpQY proteases, is shown to be major executers of this protection. Elevated Hsps are demonstrated to reduce the half-life of the oxidized proteins during conditional senescence.

(PAPER III (114)): In this paper, we used *Salmonella enterica* serovar Typhimurium LT2 to demonstrate that random mutations achieved during evolution interact such that their combined effect on fitness is mitigated (antagonistic epistasis). The levels of GroEL and DnaK were found to be elevated in lineages with many point mutations. Also, ectopic overproduction of GroEL was demonstrated to increase fitness in such strains. These data suggest that chaperones may buffer the cell against the fitness cost caused by the accumulated mutations and provides a mechanistic, physiological, explanation for antagonistic epistasis.

(PAPER IV (54)): This paper provides evidence for further links between protein oxidation and Hsps by showing that induction of the heat shock regulon in response to increased mistranslation requires oxidative modification of the malformed proteins. This is shown to be true both for cells entering stationary phase and for cells in which the ribosomes display reduced translational fidelity due to genetic manipulations, e.g. mutations in the ribosomal accuracy centre.

(PAPER V): This work established that mistranslated and oxidized proteins, in addition to affecting Hsp regulation, also affect stationary phase elevation of the transcription factor, SigmaS (σ^S) and induction of the σ^S regulon. Mechanistically, this effect of mistranslation on σ^S acts via titration of the ClpP-protease (ClpXP is responsible for σ^S degradation). σ^S is a key player in switching gene expression from growth/reproduction related activities towards those of maintenance and is essential, similar to the Hsps, to counteract protein oxidation upon entry of cells into stationary phase. We present a model for the sequence of events leading to σ^S accumulation in response to starvation.

Introduction

Why and how organisms age is a question that strikes the very heart of biology. Ageing and senescence has been referred to as a gradual decline in the cellular capacity to maintain homeostasis (122, 164, 174) that depend on both genetic and stochastic factors. Despite considerable efforts, no unifying explanation for the mechanisms of ageing exists. However, one theory that has gained in credibility is the 'free radical hypothesis of ageing'. This theory states that there is a causal relationship between damage caused by reactive oxygen species (ROS) and lifespan (72, 177, 178).

Organisms that proliferate in an oxygen containing atmosphere are continuously exposed to ROS. In addition, many stressful conditions induce the formation of ROS, but ROS is also, inevitably, produced during normal, oxidative metabolism. Hence, a large number of both constitutively expressed and stress responsive genes are involved in diverse defence systems against ROS and harmful oxidation. However, these defence systems eventually fail in fully counteracting oxidation with devastating consequences upon the individual. There are several lines of data of which each are suggestive, that together make a cumulative force, that supports the 'free radical hypothesis of ageing': (I) Oxidatively damaged macromolecules like DNA, lipids and proteins accumulate with age in all organisms examined thus far; e.g. yeast, worms, flies, and mammals, including humans (1, 110, 157, 176, 178). (II) Oxidatively modified proteins lose their functional and structural integrity (17, 110, 200, 201). (III) There is a close association between life expectancy and oxidative protein damage in house flies and bacteria (39, 175, 202). (IV) Overproduction of anti-oxidant defence systems e.g. Superoxide dismutase (Sod) prolongs lifespan by over 40% in the fruit fly, *Drosophila melanogaster* (146). Likewise, manipulations such as caloric restriction (40% reduction of food calories compared to *ad libitum* fed control group) in mice, reduces protein oxidation in mitochondria and increases lifespan (107). (V) Several gerontogenes (genes that

prolong lifespan upon altered expression) have been identified and their function in the nematode, *Caenorhabditis elegans* and *D. melanogaster* support the strong correlation between longevity and oxidative stress.

The evolutionary reason for a failure of oxidative stress defence systems to fully combat age-related oxidation of target macromolecules might be explained by the 'disposable soma theory of ageing'. This theory states that living organisms are subjected to a trade-off between growth/reproduction and maintenance. This reasoning is built upon the assumption that the resources distributed between these two activities are limited in an individual and that an elevated allocation of resources to one activity has to be 'paid-off' by a reduction in resources for the other. Thus, for a multicellular organism where the soma and germ line are distinct, reproduction will be at the cost of maintenance of the soma and long term survival (100). As stated by the free radical hypothesis of ageing, the key defence of organisms' maintenance system is protection against ROS and oxidative damage. Thus, the disposable soma theory and free radical hypothesis of aging complement each other and are certainly not mutually exclusive.

In bacteria such as *E. coli*, the distribution of resources towards growth/reproduction and/or maintenance is conditional in the sense that, as long as the environmental conditions (e.g. nutrient availability) are favourable for growth, resources are primarily diverted to growth and reproduction. The cells divide in a symmetrical fashion, evenly distributing their cytoplasmic components including damaged molecules, between the two daughters. Thus, there is no age distribution or separation between germ-line and soma and consequently no theoretical basis for a limitation in replicative potential or mandatory ageing process. Nevertheless, a recent study pointed out that cell division of the rod-shaped *E. coli* creates two daughter cells with one old pole and one new (the latter is formed at the site of division) (183). Old poles can exist from many divisions and was considered a defining character of an ageing

parent, repeatedly producing rejuvenated offspring. At first glance, old pole cells seemed to be associated with a slightly reduced growth and division rates (183), but later these deviations were argued to fall within the expected variation of length and age at division (198). Therefore there is yet no evidence for a catastrophe-like cell death through ageing in bacteria.

However, upon nutrient restriction, cell division ceases and the cells enter a growth arrested state. In these cells, a deteriorating process that has been referred to as conditional senescence sets off (134). Eventually this leads to sterility (i.e. inability of the cell to resume growth and form colonies upon nutrition) and finally to a total collapse (death) of the cell (39). This process share several features and characteristics with mandatory ageing of eukaryotic cells of multicellular organisms. For example; the time-dependent increase of intracellular oxidation damage and its target specificity, the role of antioxidants and oxygen tension in determining lifespan and, also, the regulated switch of focus from reproduction towards maintenance related activities during nutrient depletion (14, 15, 100, 135).

The experimental system

Bacterial deterioration in stationary phase has been used here as a simple model for alterations leading to cell senescence. Thus, I will give a brief introduction to the model organism and the experimental test system (stationary phase) used.

The model organism

The closely related gram negative, enteric bacteria; *E. coli* and *Salmonella enterica* serovar Typhimurium LT2 (*S. typhimurium*) have been used as model organisms in this work. Major molecular processes, e.g. DNA-replication, transcription/translation, protein management, stress protection etc. are highly conserved among biological kingdoms, and many of the studies upon which our understanding of these processes is based have been carried out using prokaryotic organisms like bacteria. In this respect, both *E. coli* and *S. typhimurium* are well established laboratory organisms. In addition, they are fast growing, have modest requirements needed for cultivation and are amenable to genetic manipulation (their whole genomes are sequenced) and molecular and physiological analyses. As outlined below, stationary phase bacteria exhibit increased oxidative damage to their proteins, a feature they share with mandatory ageing organisms. We used the simple prokaryotic model system to address the question of why such oxidation increases upon stasis, what protective devises the cell can muster against such damage, and to what extent the damage triggers alterations in gene expression, especially of the protective regulons. For this work, the capacity of the facultative anaerobe *E. coli* to grow/ reproduce and persist in the absence of oxygen has been of particular value.

Stationary phase

In natural bacterial habitats, such as the intestine for *E. coli*, nutrient availability differs vastly from almost infinite to very poor and the bacteria have to adapt

quickly to the new condition in order to compete and survive. Upon starvation, *E. coli* enters a growth arrested state, the so-called 'stationary phase' or 'stasis' (Figure 1). Since stationary phase cells have limited ability to replace damaged molecules, the demand for maintenance functions increases in this phase.

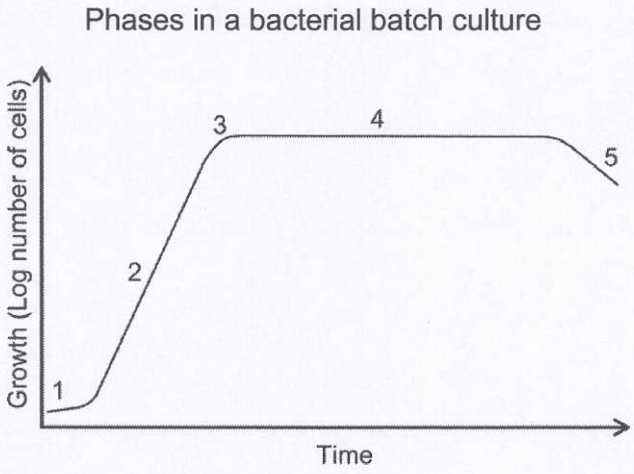

Figure 1. Schematic drawing of the phases of growth during bacterial batch cultivation. 1) Lag phase – inoculated bacteria adapt to the new media. 2) Exponential growth – reproductive growth and symmetrical cell division (in case of *E. coli*) 3) Transition phase – growth ceases due to e.g. depletion of an essential nutrient in the media. Cells go through profound rearrangements of their metabolism, gene expression and physiology in this phase. 4) Stationary phase – growth arrest and a non-reproductive phase – the 'conditional senescence' sets off and progresses with time. The cellular activities are diverted towards maintenance. 5) Death phase – systemic collapse and loss of reproductive ability. In some cases, lysis of cells.

Global regulatory networks control expression of various stress protective proteins and adjust gene expression toward maintenance related activities in

direct response to growth inhibiting environmental factors such as: high temperature, oxidative agents, osmotic fluctuation, DNA damage, and a plethora of other challenges (40, 66, 75, 85, 113). Many of these stress-specific defence systems are also induced in cells upon starvation-induced growth arrest; a phenomenon referred to as cross protection (84-86, 137, 139, 140). Cross protection leads to elevated resistance to a variety of external stresses like for example H_2O_2-treatment, heat and osmotic shock.

It should be pointed out that cellular responses to starvation, to some extent, depend on which nutrient become exhausted and also that cells in stationary phase are physiologically different over time. Nevertheless there is a general, although not identical, response in terms of stress protein production upon nutrient depletion.

The constituents and principle mechanisms as well as the targets of general stress protection systems are to a large extent evolutionary conserved and strongly analogous, e.g. among prokaryotes and eukaryotes (49, 164). This conservation suggests that starved and growth arrested cells encounter common intrinsic problems regardless of whether the cell is of prokaryotic or eukaryotic origin. Oxidative damage of cellular components by ROS seems to be one such problem (see next section)

Physiological alterations in stationary phase – an oxidative stress defence?

E. coli cells respond to aerobic carbon starvation by profound rearrangements of their metabolism in a way very similar to the metabolic swap that takes place during a shift of cells from aerobic to anaerobic conditions (140). This includes increased synthesis of specific glycolytic enzymes and strongly reduced production of enzymes in the TCA-cycle (137, 140).

The two-component regulatory system ArcA/ArcB is one of the major regulatory systems of this metabolic swap. It is activated in response to oxygen depletion when no or only poor electron acceptors are available, but it is not clear what the stimulus is for ArcA/ArcB activation upon starvation (82). However, the down-regulation of respiratory activity during starvation-induced growth arrest by ArcA is of vital importance, since deficiency of ArcA leads to high respiratory activity and poor survival of cells during carbon starvation (140). Interestingly, the shortened lifespan of *arcA* mutant cells could be counteracted by overproduction of SodA, suggesting that down regulation of respiration governed by ArcA, may be a way to decrease ROS production and, as a consequence, reduce oxidative damage during stasis (140). Thus, this might be the first line of defence of stationary phase cells to self-inflicted oxidative damage.

In addition, growth arrested cells exhibit an elevated capacity to combat ROS enzymatically. Enzymes involved in ROS detoxification systems are induced or activated upon starvation and represent a second line of protection against oxidative injuries. Among these enzymes are SodA and SodB (MnSod and FeSod respectively), that aid the dismutation of superoxide ions ($O_2^{-\cdot}$) to hydrogen peroxide (H_2O_2) and the H_2O_2 detoxifying proteins: alkyl hydroperoxide reductase (Ahp) and catalases (KatE) (45, 166).

A third line of defence against cumulative, oxidative damage in growth arrested cells encompasses proteins involved in reduction, repair or removal of damaged molecules. Examples include methionine sulfoxide reductase and glutathione reductase that work in concert with glutathione, thioredoxin, glutaredoxin, and Hsps (141). Also proteins involved in DNA and lipid repair (e.g. RecA, XthA and RuvC, and Hmp and Blc, respectively) become elevated during cellular growth arrest (24, 45, 61, 120).

Thus, similar to the ageing process of eukaryotes, oxidative damage to cellular macromolecules may be involved in the senescence process of

stationary phase *E. coli* cells (10, 39, 157, 174). In line with this notion, the mean lifespan of cells starved for exogenous carbon/energy (glucose) is around 3 to 5 days in an aerobic environment, but under anaerobic conditions, the starved cells remains 100% viable during 10 days or more (44). In addition, the accelerated death-rate of aerobically starved cells with reduced ability to combat ROS enzymatically, caused by mutations in e.g. *oxyR, katE and katG*, could be counteracted completely by externally supplied catalase or by growth under anaerobic conditions (44, 46). Hence, it seems an inescapable conclusion that oxidative damage by ROS is a major problem of starving *E. coli*.

Two major regulons defending the cell against conditional senescence

There are two major regulatory networks responsible for expression of the genes involved in stress-protection during growth arrest. Both are induced upon starvation and are under control of sigma factors: σ^S (σ^{38}) (encoded by *rpoS*) and σ^H (σ^{32}) (encoded by *rpoH*) (75, 84, 90, 91). Sigma factors bind to RNA polymerase (RNAP) and direct the polymerase to the specific promoters of the respective regulon genes (125). The σ^S system is called the general stress defence regulon whereas the σ^H system is commonly known as the heat shock regulon. We initially focused on these two regulons because in their absence cells die off more rapidly in stationary phase (accelerated senescence) and the σ^S regulon had been shown to mitigate starvation-induced protein oxidation (44, 45). In addition, Hsps have been shown to extend the life span of higher organisms when ectopically overproduced (79, 185) and we wondered whether such effect on senescence could be linked to a possible role of Hsps in counteracting protein oxidation. Below follows a description of the regulons, some of their key members, physiological functions, and regulation.

The σ^{32} regulon

The σ^{32}-dependent genes were first discovered as a set of genes induced upon a temperature upshift (188). Therefore, they are named heat shock genes and the resulting proteins are named according to their molecular mass (kDa), e.g. Hsp70. The heat shock proteins (Hsps) are strongly conserved proteins, both with regard to their function and amino acid sequence, and they are present in all organisms (49, 93).

The majority of the Hsps are chaperones and proteases involved in preventing protein injuries and in removal of damaged protein, but they also play diverse roles in unstressed cells (49, 127). They process unfolded, misfolded, damaged or aggregated polypeptide chains and support protein maturation and trafficking (127). The demand for these functions increases during environmental stress and stress-induction of Hsp genes is intimately associated with the accumulation of aberrant proteins in organisms from all the branches of the evolutionary tree, PAPER I, PAPER IV (10, 54, 127, 191).

DnaK /Hsp70

The most well characterized Hsps are the ubiquitous members of the conserved and large Hsp70 family of ATP-dependent molecular chaperones. All Hsp70 proteins are structurally similar; they all contain an actin-like N-terminal ATPase domain of approximately 45 kDa (50, 51), an approximately 15 kDa substrate-binding domain (SBD), and a 10 kDa C-terminal domain that is involved in interaction with co-chaperones and probably have other functions as well (55, 210). Hsp70s participate in a wide range of activities such as, refolding of stress-denatured soluble proteins, resolubilization of aggregated proteins, native protein folding during protein synthesis, translocation of proteins across membranes, assembly and disassembly of protein complexes and they also regulate signal transduction pathways by controlling the stability and activities

of protein kinases and transcription factors (47, 191). Substrate proteins of the Hsp70 chaperone machinery usually expose hydrophobic amino acid residues, normally hidden in protein structure and these hydrophobic regions are recognized by the SBD of Hsp70 that constitutes a hydrophobic pocket (108, 210). Binding and processing of target proteins depend on ATP-hydrolysis and interaction with co-chaperones, i.e. J-domain proteins (JDPs/Hsp40s) and with nucleotide exchange factors, both of which are parts of the Hsp70 chaperone system (47). JDPs are a heterologous group of multidomain proteins, defined by the highly conserved J-domain, essential for stimulating ATP hydrolysis of Hsp70s (22).

E. coli contains three *hsp70* genes encoding DnaK, HscA (heat shock cognate A) (Hsc66) and HscC (Hsc62) and six Hsp40 proteins (DnaJ, CbpA, DjlA, HscB (Hsc20), YbeV (Hsc56) and YbeS (81). Hscs are proteins with similar properties and functions as the Hsps, but are not inducible by temperature upshifts. DnaK is the major Hsp70 and the most well described of all Hsp70 proteins. DnaJ is the main co-chaperone of DnaK, but CbpA and DjlA has also been shown to interact with DnaK. HscA together with HscB has specialized functions in the biosynthesis of iron-sulfur proteins (171), while HscC, in cooperation with Hsc56 negatively modulates the activity of Sigma70 (σ^{70}), the housekeeping sigma factor (6, 205).

The basic principles of the major *E. coli* Hsp70 chaperone system (DnaK/J/GrpE) substrate interaction cycle (based upon the references (11, 47, 71, 173)) are: (1) In the ATP-bound state DnaK has low affinity for target peptides. (2) ATP hydrolysis, which is highly accelerated by transient association with the Zinc-containing (48) co-chaperone DnaJ in the presence of substrate, converts DnaK to a substrate-high-affinity conformation. Since there are at least 10 times more DnaK than DnaJ in the cell, this step is rate limiting (13). DnaJ on its' own, associates with certain substrates (e.g. σ^{32}), before

binding to DnaK. (3) Substrate release from DnaK after ATP hydrolysis is triggered by the nucleotide exchange factor GrpE.

Unfolding or refolding of a denatured protein might involve several cycles of binding and release of the substrate and also cooperative shuttling of a substrate between different chaperone systems. Such substrate shuttling has been demonstrated to occur for example between the DnaK/J/GrpE and GroEL/ES (Hsp60/10) chaperone systems in *E. coli* (124) Together with ClpB, the DnaK/J/GrpE system also take part in resolubilization of aggregated proteins. ClpB is a chaperone that belongs to the Clp/Hsp100 family of the AAA+ (ATPases associated with diverse cellular activities) protein superfamily. Other members of this family are for example Hsp104 (that is essential for the acquisition of thermotolerance in yeast), Hsp101 in the plant *Arabidopsis thaliana* and Hsp78 in mitochondria (103, 147, 152).

GroEL/Hsp60

Besides the DnaK/J/GrpE machinery, the GroEL/ES barrel-shaped, ATP-driven chaperonin is essential for proper protein folding in *E. coli*. Deletion of either *groEL* or *groES* in a *dnaK* mutant strain background results in extensive protein aggregation (48) and together these two complexes constitute the major chaperone systems of *E. coli* (15-20% of total protein at 46°C) (7). GroEL/ES folds many unrelated polypeptides and belongs to the Group I chaperonins found in bacteria, mitochondria and chloroplasts, while Group II chaperonins is found in the cytosol of eukaryotes such as yeast (CCT) and archeae (170, 192). GroEL/ES is the best characterized chaperonin and it is composed of two rings, each of which consists of seven subunits, arranged back to back (170). Substrates are trapped to one of the GroEL (*cis*) rings via hydrophobic interactions after which binding of the co-chaperone GroES (a single heptameric ring of 10 kDa) forms a lid of the cavity. Together with binding of ATP, this induces strong conformational alterations that encapsulate and promote folding

of the substrate peptide in the hydrophilic cavity. ATP is hydrolysed and this primes release of GroES from GroEL. Upon binding of ATP to the opposite GroEL (*trans*) ring, GroES, the product polypeptide and ADP are discharged, leaving GroEL ready for another round of substrate interaction.

Lon

ATP-dependent proteases are responsible for most protein degradation in cells (26, 62). The ATPase domain (that belongs to the AAA+ superfamily) and the proteolytic domain of these proteases can either originate from separate assembled subunits or be contained within the same polypeptide chain. The *E. coli* Lon was the first ATP-utilizing protease to be identified and it has since been found in most organisms (18, 193). ATP is not an absolute requirement for the enzyme, but protein degradation is stimulated up to nine-fold by ATP (18). Lon is an oligomeric multidomain protein with a highly conserved Ser-Lys catalytic dyad in the active site (21). Deletion of *lon* is detrimental for many species, since Lon specifically controls the stability of key proteins (62). For example, *E. coli* cells lacking *lon* are sensitive to DNA damage and UV light due to stabilization and accumulation of the cell division inhibitor SulA (199). Further, the transcriptional regulator of capsule production, RcsA, is also stabilized in *lon* mutants leading to excess capsular polysaccharide production and a characteristic, mucoid phenotype (199). Lysogeny of certain bacteriophages and the anti-toxin of the toxin/anti-toxin (TA) systems in *E. coli* are also controlled by Lon (64, 67).

Lon is the primary protease degrading misfolded and aberrant proteins in the *E. coli* cytosol and extensive protein aggregation occurs in its absence upon a heat shock (161, 190). Since aberrant proteins are intrinsically sensitive to carbonylation this is in line with the results demonstrating that carbonylated proteins accumulate dramatically in growth arrested *lon*-mutants (section 'Protein oxidation – Repair and removal', PAPER II (53)).

In *E. coli*, Lon has been demonstrated to degrade ribosomal proteins after a nutritional down-shift (amino acid starvation); a process important for adaptation to the starvation condition by providing the cell with an internal pool of amino acids (105). This process is highly stimulated by stress-induced accumulation of inorganic polyphosphate (polyP) that bind the ATPase domain of Lon (105). In addition, the ATPase activity of Lon is stimulated by non-specific binding of the ATPase domain to DNA and polyP inhibits such Lon/DNA interaction in a competitive manner (27, 133) thus indicating a complex regulatory network of Lon activity.

ClpAP and ClpXP

The Clp proteases are, after Lon, the major cytosolic proteases in *E. coli*. Together, Lon and Clp proteases are responsible for 70-80% of energy-dependent proteolysis (119). Ortologs to the Clp proteins are found in most organisms (26, 52). In contrast to Lon, the Clp proteases contain the ATPase and the proteolytic activities on separate subunits. The proteolytic subunit ClpP is a serine protease where two heptameric rings form a proteolytic chamber with a narrow axial pore for substrate entry in each end. Small peptides can be hydrolyzed by ClpP, but larger peptides cannot enter the narrow pore without the assistance of an AAA+ superfamily chaperone, e.g. ClpA or ClpX. Both ClpA and ClpX are hexameric ring-shaped chaperones that upon binding to ClpP, form the ATP-dependent proteases ClpAP and ClpXP. In contrast to ClpX and ClpP, ClpA is not under control of a σ^{32}-dependent heat shock promoter (94), but it is required for optimal recovery from exposure to high temperature (187).

ClpAP degrades a variety of proteins; e.g. proteins with abnormal N-terminal amino acid residues according to the N-end rule (189), the TA system protein MazE, the P1 phage-encoded RepA, abnormal canavanine containing proteins, and ClpA itself (26, 62, 94). *In vitro* experiments suggest that substrate

specificity of ClpAP is modulated by interaction with ClpS, a small (106 amino acids) protein, encoded by a gene immediately upstream of *clpA*. For example, ClpS redirects the ClpAP proteolytic activity from degradation of SsrA-tagged polypeptides and ClpA itself towards aggregated or oligomeric proteins (41).

The most important protease responsible for *in vivo* degradation of SsrA-tagged polypeptides is ClpXP. ClpX also directs ClpP proteolytic activity towards processes involved in DNA damage repair and stationary-phase gene expression (165, 182). Five classes of ClpX-recognition signals has been discovered and it has been suggested that some of these signals might be hidden inside protein structure and only become exposed upon misfolding (52). Unfolding of protein substrates by ClpX probably occurs by iterative mechanical force and consumes four times the ATP required for translocation into ClpP and thus constitutes the rate limiting step for protein degradation (95).

ClpXP/AP plays important roles in stationary phase adaptation and survival of growth arrested *E. coli* cells in several ways. ClpXP is the protease that carries out SprE(RssB)-dependent degradation of the stationary phase transcription factor σ^S, and both ClpAP/XP specifically degrade numerous growth phase regulated proteins (34, 195). Absence of these proteases reduces both viability during growth arrest and the ability to resume growth upon addition of nutrients (151, 209)

HslVU/ClpYQ

The Hsp protease HslVU (also called ClpYQ) is a bacterial homolog to the eukaryotic proteasome (167). The chaperone unit, HslU, share 50% sequence homology with ClpX, while the proteolytic subunit, HslV, display sequence similarity to the β-subunit of the 20S proteasome and similarly contains a catalytic N-terminal threonine residue. HslU forms a single hexameric ring that bind the HslV dodecamer consisting of two stacked hexameric rings (31, 167). HslVU can partly compensate for a deletion of *lon*, i.e. overproduction of

HslVU suppresses both the sensitivity to DNA damage, and excess capsular polysaccharide production, implying an overlap in substrate specificity among the proteases (92, 96). Similar to Lon and the proteasome, HslVU also participates in degradation of abnormal and oxidized proteins (19, 29, 92), and deletion of either Lon or HslVU augments protein carbonylation in growth arrested *E. coli* cells as described in section 'Protein oxidation – Repair and removal', PAPER II (53).

Regulation of Hsps

In *E. coli*, the cytoplasmic Hsps are under positive control of σ^{32}, that binds to RNAP and directs the RNAP to specific heat shock promoters (68). Transcription by RNAP-σ^{32} is negatively modulated by an Hsp feedback loop, involving the DnaK/J/GrpE chaperone system that binds σ^{32} and eventually directs it to proteolysis (190, 191). The major protease in this pathway is the σ^{32}-dependent zinc-dependent metalloprotease FtsH, but HslVU, other Clp proteases and Lon has also been reported to contribute to σ^{32} degradation (92). The DnaK/J chaperones also recognize and bind to hydrophobic amino acid patches exposed by aberrant and denatured proteins. Since the levels of DnaK/J are limiting in vivo (191, 206), increased levels of aberrant proteins consequently renders σ^{32} more stable by sequestering of the DnaK/J system (57, 68). Therefore, this model of Hsp regulation is referred to as the 'titration model' and this regulatory mechanism constitutes a sensitive and tight control system that adjusts the Hsp levels to precisely fit the cellular demand under a specific condition (Figure 2).

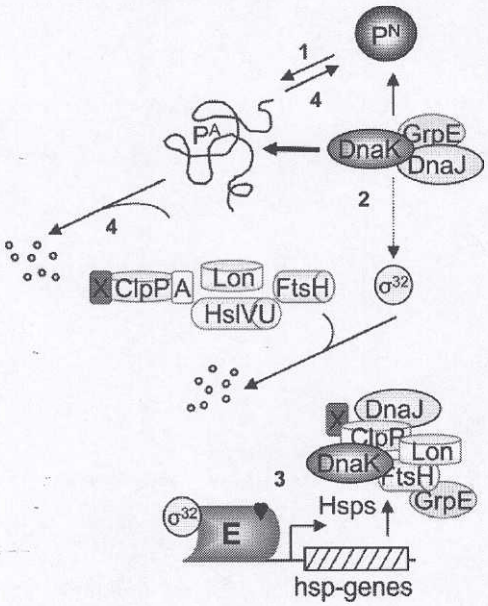

Figure 2. Schematic representation of the 'titration model' for Hsp regulation. (1) Elevated levels of substrates (aberrant proteins, P^A) sequester the DnaK/J/GrpE chaperone system (2) such that its negative effects on σ^{32} is alleviated (68, 191). σ^{32} binds RNA polymerase (E) and (3) directs the polymerase to heat shock promoters, resulting in increased production of Hsps. Upon successful refolding/degradation of the aberrant proteins (4) by the Hsps, the DnaK/J/GrpE chaperone system again binds σ^{32} and hereby strongly reduces Hsp production. This feedback loop provides an efficient mechanism for tight regulation and a fast shut off of excess Hsp production. For further details upon Hsp regulation, see text.

There are also additional regulatory pathways of Hsp expression in *E. coli*. For example, a temperature-upshift rapidly increases translation of σ^{32} by destabilization of the *rpoH* mRNA secondary structure, thereby increasing the ribosomal accessibility to the translation start site (128). In addition, the nucleotide exchange factor GrpE has been demonstrated to work less efficiently

during a temperature-upsift, leading to a higher fraction of DnaK being bound to ADP. DnaK-ADP has high affinity for substrates (169) and this altered activity of GrpE may ensure a very rapid and sensitive increase in Hsp production, via stabilization of σ^{32}, that precedes protein unfolding and σ^{32} synthesis (169).

Besides specific stress-induction, Hsp gene expression is induced upon cellular growth arrest in both prokaryotes and eukaryotes (84, 86, 118, 127, 136). In *E. coli* cells, such induction can be counteracted by several means that reduce the production of aberrant proteins and/or oxidatively damaged proteins. Among these are; increased translational fidelity, overproduction of Sod and omission of oxygen, PAPER I, PAPER IV (10, 43, 54). The latter deserve some extra attention, since translational errors, such as nonsense suppression and frameshifting, were found to be substantially elevated in cells cultivated anaerobically, PAPER IV (54). Yet the demand for Hsp function is significantly lower in these cells than in those propagated aerobically. This strongly suggests that oxidative modifications of misfolded proteins promote a further loss of the proteins structural integrity and consequently increased exposure of hydrophobic surfaces. Such an increase in the target sites for the DnaK/J/GrpE chaperone system, render these proteins more efficient in sequestration of the DnaK chaperone system and stabilization of σ^{32}. This is further supported by the fact that ribosomal ambiguity mutations (*rpsD*) only enhance Hsp gene expression in cells propagated aerobically, PAPER IV (54).

Another possible regulatory mechanism of Hsp expression in *E. coli* involves detrimental oxidation of DnaK itself. Such damage would also increase Hsp gene expression via stabilization of σ^{32}, provided that oxidized DnaK is non-functional. Indeed, a larger fraction of DnaK shows signs of structural aberrancy under aerobic conditions, PAPER IV (54, 184, 197). In line with this, a recent study shows that DnaK is reversibly inactivated upon heat stress in the presence of H_2O_2 (197). This inactivation was linked to H_2O_2 significantly reducing cellular ATP-levels leading to nucleotide deprivation of the N-terminal

ATPase domain of DnaK, which, as a consequence, becomes thermolabile and unfolded. It is noteworthy that in vitro refolding of the inactivated DnaK required the presence of a reducing agent; e.g. stress removal and addition of ATP was not enough, implying that the unfolded domain is oxidatively modified (197).

Other Hsp70 proteins in distantly related organisms are, similar to DnaK, intrinsically sensitive to carbonylation (23, 89, 157). This might point to a role of Hsp70 proteins in oxidation sensing/signalling that enables a rapid and direct elevation of Hsp levels in response to oxidative stress.

Hsps in disease and ageing

The necessity of the Hsps function for maintaining protein as well as organismal homeostasis is underlined by the fact that altered expression of Hsps is associated with several diseases such as ischemia and reperfusion damage, cardiac hypertrophy, fever, inflammation, metabolic diseases, infection, cell and tissue trauma and cancer (126). Furthermore, studies have demonstrated that epistatic manipulation of Hsp levels can affect the course of a disease-related injury. For example, hearts isolated from transgenic mice overproducing either human or rat inducible Hsp70, were strongly protected against ischemia and reperfusion damage (117, 149). Such damage involves disruption of protein homeostasis and oxidative injuries caused by oxygen radicals produced during reperfusion. Hsp70 might bind the misfolded and denatured proteins that appear during ischemia and promote their refolding and renaturation during reperfusion (117, 149). We have shown that misfolded proteins are sensitive targets to oxygen radicals, PAPER I (10, 42) and as demonstrated in Fredriksson *et al.*, (2005), PAPER II (53), overproduction of the prokaryotic Hsp70 homologue DnaK, confers a general protection against protein oxidation in *E. coli* cells upon growth arrest caused by glucose deprivation; a condition known to elevate protein aberrancies, PAPER I (10). In addition, the Hsps most likely play

important roles during the course of several age-related neurodegenerative diseases, e.g., Alzheimer's and Parkinson's disease; disorders involving accumulation of aggregated and oxidized proteins (158).

Hsps also seem to be implicated in the ageing or senescence process of a growing number of organisms. For example, the amount of Hsp70 mRNA declines with age in various rat tissues (16) and this was found, at least for liver and brain, to be a consequence of reduced activity of the heat shock transcription factor; HSF-1 rather than decreased HSF-1 levels (168). Aged humans also exhibit altered Hsp levels. For example, both Hsp70 and Hsp60 levels in serum have been shown to decrease with age (154).

Experiments have repeatedly demonstrated that Hsp70 can affect survival and its levels are also affected by the oxidation status of the organisms. For example, elevated levels of Hsp70 can prolong lifespan in transgenic flies (185), and the worm *C. elegans* (204). In line with this, mild heat stress early in life of flies lead to elevated levels of Hsp70, improved longevity, and also enhanced capability to induce *hsp70* and survive potentially lethal heat stress later in life. On the other hand, flies selected for longevity exhibited a reduced ability to produce Hsp70 in response to elevated temperature (77). In *C. elegans,* decreased transcription of the heat shock genes due to reduced activity of HSF-1, causes a rapid-aging phenotype and shortened lifespan (59, 129), while overproduction of HSF-1, conveys heat and oxidative stress resistance, and a 40% increase in lifespan (79). This effect was at least in part due to elevated expression of small Hsps (sHsps). A link between the normal ageing process and the diseases of ageing was also demonstrated, since reduced expression of sHsps was found to accelerate the onset of aggregation of Huntington's like polyglutamine-repeat proteins expressed in *C. elegans* (79). In *D. melanogaster*, overexpression of sHsps has been demonstrated to likewise extend lifespan and increase resistance to oxidative as well as thermal stress (104, 130). Specifically, overproduction of Hsp22 in the mitochondria of motorneurons was

demonstrated to increase the mean lifespan (30%) and resistance to oxidative stress (35%) (131). This is comparable to the 40% increase in lifespan obtained by transgenic expression of human *sod1* in the motorneurons of flies (146). Conversely, prevention of Hsp22 synthesis reduces lifespan (130).

Thus, an increasing number of studies points to a close connection between Hsps and protein oxidation, ageing and age-related disorders. The Hsps evidently have a role in cellular resistance against oxidative stress (79) and are increasingly expressed during oxidant exposure (5). In *E. coli* cells, stasis-induced protein carbonylation is drastically mitigated by overproduction of the Hsps and DnaK is one key factor in this defence, PAPER II (53). In addition, Hsps are themselves targets of carbonylation, PAPER II (25, 53, 89) and it is conceivable that such damage to these cyto-protective proteins may eventually lead to a total collapse of the cell/organism.

Hsps and buffering against accumulated mutations

The strong link between protein aberrancy and Hsps and the ability of the Hsps to prevent accumulation of misfolded proteins, raises the possibility that these functions of the Hsps may have important consequences also in an evolutionary perspective. A key parameter in evolutionary biology is the relationship between the number of randomly accumulated mutations, e.g. point mutations, in a genome and fitness (98, 101). Point mutations may lead to increased protein misfolding and hence to reduced enzyme function and consequentially to reduced fitness. However, it is possible that increased numbers of point mutations also elevates Hsp production by sequestration of σ^{32} by the mutated proteins (see section 'The σ^{32}-regulon – Regulation of Hsps'). The Hsps may 'buffer' for the phenotypic consequences of the mutated genotype, i.e. enzymes carrying mutations (e.g. amino acid substitutions) will still be functional due to, for example, a high chaperone activity that continuously refold unstable domains of the protein. Also, enhanced proteolysis of misfolded and aggregation

prone polypeptide chains may prevent accumulation and oxidation damage of those protein species.

Using *S. typhimurium* we demonstrated that random mutations achieved during evolution interact such that their combined effect on fitness is mitigated (Antagonistic Epistasis), PAPER III (114). The levels of GroEL and DnaK were found to be elevated under these circumstances and ectopic elevation of GroEL was found to buffer against the fitness cost caused by accumulated mutations (114). The elevated levels of Hsps in response to accumulated mutations provide a mechanistic, physiological, explanation for antagonistic epistasis.

In addition, based on previous results and the data of this thesis (PAPERS I, II, and IV), demonstrating that aberrant proteins are more susceptible to oxidation and that Hsp chaperones are regulated by oxidation and involved in mitigating protein oxidation, it is possible that the buffering effects of Hsps on accumulated mutations are more critical and/or efficient during aerobic than anaerobic conditions. This remains to be elucidated.

The σ^S-regulon

The σ^S-dependent genes are strongly induced when *E. coli* cells are exposed to various stress conditions as summarized in figure 3 (63, 75, 150, 194).

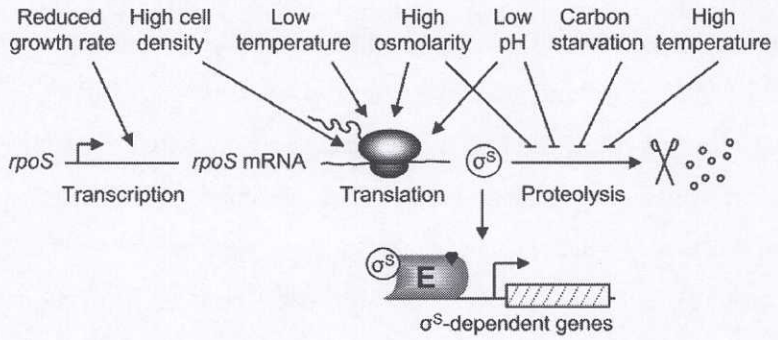

Figure 3. Schematic representation and summary of the complex and multifaceted regulation of σ^S, adapted from (75). σ^S is regulated at both the transcriptional, translational and post-translational level depending on the specific stress condition. σ^S protein binds RNAP and directs the polymerases' transcriptional activity to expression of σ^S-dependent genes.

σ^S is a key player in the switch of the cellular gene expression from growth/reproduction related activities towards those of maintenance and about 10% of the *E. coli* genes are under direct or indirect control of σ^S (194). Cells lacking functional σ^S caused by mutations in *rpoS* are poor survivors of stressful conditions as well as during growth arrest (106).

It is not clear which members of the σ^S-regulon are most important in defeating senescence, but since σ^S-deficient cells have high levels of oxidatively damaged proteins (43, 44) and *rpoS* mutants fail to express oxidative stress defence genes such as superoxide dismutase (*sodC*) and catalase (*katE*), such

stress defence proteins are likely candidates. The link between σ^S-mediated oxidation protection and growth arrest survival has been supported also by experiments in *S. typhimurium* (186) that also showed that σ^S were assisted and complemented by another sigma factor, σ^E, in this role. The transcription factor σ^E regulates the expression of extracytoplasmic chaperones and proteases, many of which also participate in the biogenesis of the cell envelope in the absence of stress (162). Transcription of σ^E-regulated genes is triggered by misfolded proteins in the periplasm, severe heat stress, and by growth arrest (132, 162). Mutants lacking both σ^S and σ^E loses viability almost immediately upon growth arrest under aerobic conditions, but survival of these mutants is completely preserved during anaerobic growth arrest (186). This reinforces the argument that oxidative damage is a major obstacle for prokaryote survival of growth arrest and also that σ^S has an important role in preventing such damage.

During growth/reproduction, σ^S is a very unstable protein with a half-life < 1 minute. The majority of the genes expressed during exponential growth, i.e. genes involved in substrate uptake, DNA replication, cell wall/membrane biosynthesis, ribosome production and also most genes of the protein synthesizing system, require the sigma factor, σ^{70} (encoded by *rpoD*) for transcription initiation (113, 125). However, upon nutrient limitation, σ^S is drastically stabilized (207) and transcription by RNAP primed with σ^S increases, at the expense of σ^{70}-dependent gene expression (75, 88). This results in up-regulation of stress-defensive and other maintenance-related genes, while expression of growth/reproduction related genes decreases (75). A key process for this metabolic switch is regulated σ^S proteolysis (207). The protease ClpXP and the two-component response regulator RssB, a specific σ^S recognition factor, are essential for this process (116, 148, 151).

Recent data demonstrate that protein oxidation is involved in the stabilization of σ^S in growth arrested cells (PAPER V) and that this can be

linked to ribosomal fidelity. Mutations causing high translational accuracy, drastically attenuate induction of the *rpoS* regulon and prevents stabilization of σ^S upon starvation. In contrast, mutations augmenting translational errors cause elevated levels of σ^S. Altered translational fidelity affects σ^S stability independently of the σ^S recognition factor RssB. Instead, protein stability measurements and genetic suppression suggests that σ^S becomes stabilized upon starvation as a result of ClpXP sequestration and this sequestration requires oxidative modifications of the mistranslated proteins (Figure 4).

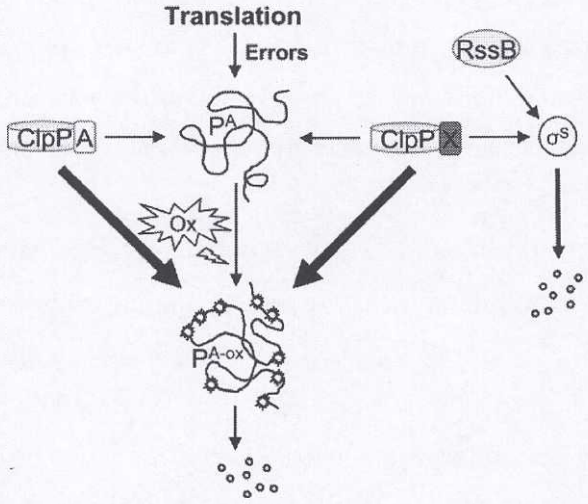

Figure 4. Schematic model of stabilization of σ^S via increased protein oxidation. Increased translational errors result in enhanced production of aberrant proteins (P^A), and as a consequence in elevated levels of oxidized proteins ($P^{A\text{-}ox}$). The oxidized proteins efficiently sequester the ClpAP and ClpXP proteases leading to stabilization of σ^S, independently of RssB.

In addiditon to σ^S, maintenance-related gene expression and activities also requires the alarmone ppGpp that is synthesized upon carbon and amino acid

starvation (113). Cells deficient of ppGpp are unable to switch from growth/reproduction to maintenance related gene expression and die quickly upon starvation (113). This may, at least partly, be due to that ppGpp is essential for both σ^S-dependent and σ^{32}-dependent activities (113). Mechanistically, this can be explained by the fact that ppGpp, binding directly to the RNA polymerase, lowers the affinity of the polymerase to σ^{70}, while the affinity for σ^S and σ^{32} is increased (Figure 5) (88). Interestingly, ppGpp-deficient cells also have high accumulation of oxidized proteins, further establishing the role of global alterations in gene expression upon starvation in mitigating oxidative damage (Manuel Ballesteros, personal communication)

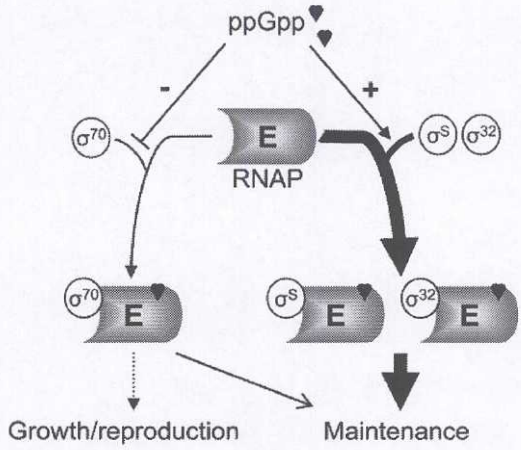

Figure 5. During starvation, production of the alarmone ppGpp (♥) is increased, resulting in strongly elevated transcription of maintenance related genes by RNAP-σ^S and RNAP-σ^{32} at the expense of growth related RNAP-σ^{70}-dependent genes. However, some genes involved in maintenance are under control of σ^{70} and expression of those is also elevated in a ppGpp-dependent manner (see Magnusson *et al* for a review (113)). The trade-off between reproduction and maintenance is mechanistically linked to the fact that RNAP is limiting and ppGpp affects sigma factor competition such that elevated ppGpp favours σ^S and σ^{32} binding to RNAP.

Taken together, oxidized proteins seem to enhance both σ^{32} and σ^{S}-dependent gene expression via sequestration of Hsp chaperones and proteases upon entry of cells into stationary phase. This leads to a precise adjustment of gene expression such that the production of Hsps and antioxidant enzymes is in equilibrium with the degree of oxidative damage (Figure 6).

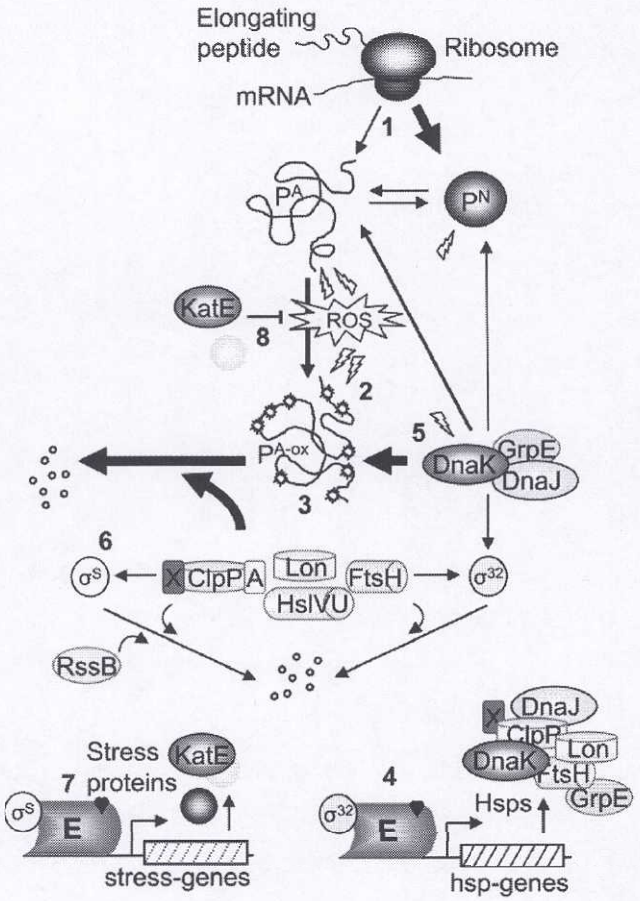

Figure 6. Schematic representation of events linked to irreversible protein oxidation (carbonylation) in growth arrested *E. coli*. 1) Mistranslation increases as a consequence of starvation and the ribosomes produce aberrant proteins (P^{A}) which

themselves are not good substrates for the DnaK/J/GrpE chaperone system, unless they 2) become oxidized by reactive oxygen species (ROS). 3) The oxidized proteins ($P^{A\text{-}ox}$) are high-affinity substrates for the DnaK/J/GrpE chaperone system that presumably direct them to proteolysis since carbonylated proteins cannot be repaired. 4) This sequesters the DnaK/J/GrpE chaperone system and the proteases leading to stabilization of σ^{32} that bind RNA polymerase (E) and directs the polymerase to the Hsp genes resulting in increased Hsp production. 5) In addition, DnaK itself can become oxidatively damaged and unable to bind σ^{32}. 6) σ^{S} becomes stabilized via titration of ClpP and 7) similarly binds RNAP leading to 8) elevated expression of general stress defence proteins, e.g. KatE, other antioxidant enzymes, and maintenance genes. The mechanisms described, ensure that irreversibly damaged proteins, via oxidation, are rapidly delivered to proteases and are not incorporated into cellular machines involved in information transfer such as DNA/RNA polymerases and ribosomes, and at the same time the general stress defence and protein protection capacity of the cell become adequately elevated.

Protein oxidation

In view of the fact that the major stationary phase regulons described in this thesis is involved in protecting the cells against protein oxidation, the generation of such damage and its consequences for the cell deserves some special attention.

Proteins are the major constituents of most biological systems whether this are at the tissue, biological fluid, or cellular level (36), and they participate in almost every cellular process. Hence they are absolutely essential for biological life and a general increase in damage of proteins most likely makes cells more vulnerable to (accidental) death. Everyday wear and tear exposes proteins to a wide variety of potentially damaging events and factors, e.g. ROS, mechanical and chemical injuries, temperature- and pH-changes. ROS appears to be of special interest since a substantial number of reports point out various types of oxidative protein damage as being important in the process of ageing and senescence (10, 39, 157, 174). In addition, erroneous *de novo* protein synthesis and misfolding affect protein quality (62).

ROS-production can be induced by many stressful conditions and via many pathways (15, 164). However, ROS is also a bi-product of normal aerobic metabolism formed by incomplete reduction of molecular oxygen (O_2) to water (H_2O) (164). There are many types of ROS generated within a cell and their reactivity and stability differs vastly. The most unstable and reactive and hence most detrimental ROS is the hydroxyl radical $OH^{-}\cdot$ (164). While cells are equipped with multiple protection systems for the less reactive ROS, e.g. singlet oxygen, superoxide ions ($O_2^{-}\cdot$), hydrogen peroxide (H_2O_2), $OH^{-}\cdot$ thwarts the antioxidant systems and reacts quickly with the nearest target at rates limited by diffusion (164). Reaction with ROS by cellular molecules can lead to the formation of many other types of radicals that in turn react further, thus exacerbating the initial damage.

Damaging protein oxidation – carbonylation

Oxidative reactions, such as disulfide bridge formation between thiol-groups (-SH) on cysteine residues, are essential during native protein folding but also for regulation of protein activity. For example, intramolecular disulfide bridge formation is required to induce homodimerisation and thereby activation of the chaperone holdase Hsp33 (65, 83), which is important during heat and oxidative stress (197). Also the transcription factor OxyR that regulates expression of oxidative stress defence genes (e.g. *katG, gorA*) is regulated by a similar mechanism, involving internal disulfide bridge formation (208).

However, ROS cause a wide variety of illegitimate modifications on proteins. Among these are; hydroxylation of aromatic groups and aliphatic amino acid side chains, nitration of aromatic amino acid residues, nitrosylation of sulfhydryl groups, sulfoxidation of methionine residues, chlorination of aromatic groups and primary amino groups, and carbonylation (179), of which the latter, carbonylation, has gained attention in ageing and disease research.

Protein carbonylation, as the name indicates, is an oxidative formation of carbonyl groups (aldehydes and ketones), on primarily lysines, arginines, prolines and threonines (179), but can also target cysteines, histidines and lysines via lipid peroxidation and non-enzymatic glycation/glycoxidation (179). The quantitatively most abundant products of the carbonylation reaction are glutamic semialdehyde from arginine (Figure 7 A) and proline, and aminoadipic semialdehyde from lysine (156). Being structurally different from their original amino acids, these compounds alter the chemical and structural properties of a protein (110, 174).

In contrast to other oxidative modifications of proteins, for example, methionine sulfoxide and cysteine disulfide bond formation, carbonylation is relatively difficult to induce and it is an irreversible modification (33). Carbonylation is thus detrimental to protein structure and enzymatic function

(111, 200, 201) and a carbonylated protein cannot be repaired; it is sentenced to degradation (42) or aggregation (17).

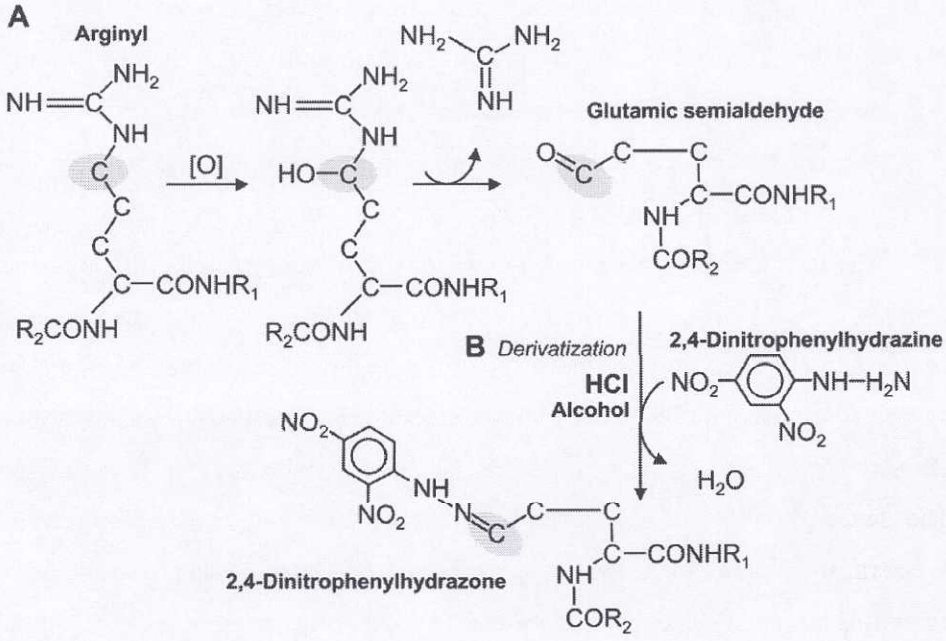

Figure 7. Schematic overview of carbonylation and derivatization of a protein amino acid (arginine) side chain, adapted from (97, 138, 155). A) Formation of one of the quantitatively most abundant products of the carbonylation reaction: glutamic semialdehyde from an arginyl residue via oxidation. B) For detection, the carbonyl group is derivatized by reaction with 2,4-dinitrophenylhydrazine to 2,4-dinitrophenylhydrazone that can be detected by specific antibodies.

A link between protein carbonylation and enzyme activity/stability was first established in studies on the bacterial glutamine synthetase activity (110). Glutamine synthetase is rapidly degraded in *E. coli* cells upon nitrogen starvation in a two-step-process: 1) Upon substrate limitation, the metal-

nucleotide binding pocket of the enzyme's active site becomes available for oxidative attack leading to inactivation of the catalytic activity of the enzyme, decreased metal binding capacity, loss of histidine-269 and increased carbonyl content (of which arginine-344 in the active site is one target) (32, 112, 159, 160). 2) Subsequent loss of an additional histidine residue results in proteolytic degradation of the modified enzyme (159).

Protein carbonylation is commonly used today as a diagnostic bio-marker of oxidation damage on proteins (33). Carbonyl groups formed on oxidatively damaged proteins can be easily detected if first derivatized to 2,4-dinitrophenylhydrazone (DNP-hydrazone) by reaction with 2,4-dinitrophenylhydrazine (DNPH) (Figure 7 B). The carbonyl content of whole protein extracts can then be determined spectrophotometrically, or immunochemically using antibodies specific to the DNP moiety of the protein. The latter method, if combined with SDS-PAGE gel electrophoresis and Western blotting, allows the carbonyl content of individual proteins to be analyzed.

Protein carbonylation and ageing

Accumulation of carbonylated proteins has been demonstrated to occur during many disease conditions such as cancer, cataractogenesis, sepsis and also the age-related, neurodegenerative, Alzheimer's and Parkinson's diseases (33, 110). Protein carbonylation also increases with ageing and senescence in all organisms examined thus far; e.g. yeast, worms, flies, and mammals, including humans (14, 110, 157). In general, the carbonyl content increases at a rather modest rate during the first two thirds of the lifespan, but during the last third the rate is dramatically elevated (1, 60, 110, 143, 175, 181). This corresponds to the post-reproductive age of the organisms analyzed.

It is not clear why carbonylated proteins accumulate with age. The classical antioxidants including superoxide dismutases, catalases and peroxidases seem to be key members of the cellular defence against protein carbonylation (138). Yet, there is no clear link between reduced activities or abundance of specific members of the various oxidant defence systems and increased oxidation in ageing cells. For example, catalase activity was demonstrated to either increase or decrease with age in a tissue specific manner and did not coincide with elevated protein carbonylation (176). Similarly, other antioxidants have been found to increase or decrease with age in the same tissue (9, 87). On the other hand, when the ability to withstand X-irradiation-induced oxidative stress was determined for whole body (house flies (2)) and various tissue-homogenates (176), oxidative damage increased linearly the older the originate of the flies/tissues.

In growth arrested *E. coli*, the oxidant defence systems are elevated, yet the cells fail to prevent stasis-induced protein oxidation (43, 44, 76, 118). However, sterile cells that appear in subpopulations of starving *E. coli* cultures have been demonstrated to display decreased Sod activity and levels but increased KatE activity, implying an imbalance in their oxidant defence (39). These sterile cells also had strongly elevated levels of carbonylated proteins compared to the healthy fraction. In addition, it is possible that senescence can be caused by increased oxidative stress as a consequence of oxidative damage of the anti-oxidant enzymes themselves, but no evidence for this has yet been presented in the bacterial model system for conditional senescence.

Another possible mechanism of increased carbonylation with age is elevated ROS production. Indeed, there are some evidence for a correlation between increased protein carbonylation and elevated mitochondrial ROS production in ageing organisms (109, 153). Among the suggested causes of this age-related inceased ROS production are mitochondrial dysfunction, increased intracellular

abundance of free iron, etc. (15, 38). In line with this notion, it has been suggested that carbonylated proteins accumulate in growth arrested cells (e.g. *E. coli* or somatic G_0 cells) because such cells has an ongoing respiratory activity and therefore high production of ROS, but little or no ability to dilute any damage via *de novo* macromolecular synthesis and/or cell division. This idea is in line with the 'rate-of-living' hypothesis and in its simplest form it states that the higher the rate of respiration, the higher the oxidative damage and the shorter the lifespan. However, experiments in *E. coli* and yeast G_0 cells do not support this notion, since there was no strict correlation between high respiratory activity, protein carbonylation and lifespan, PAPER I. (4, 10) In contrast to carbon- and nitrogen-starved *E. coli* cells, cells starved for phosphate maintain a high metabolic activity for extended periods of growth arrest, PAPER I (10, 61). However, while C- and N-starved cells rapidly accumulate high levels of carbonylated proteins, only a modest increase occurs in P-starved cells. Also, cell-viability (culture half-life) correlates inversely with protein carbonylation, but not with metabolic activity, i.e. P-starved cells survive much better during growth arrest compared to C- and N-starved cells, PAPER I (10). In addition, protein carbonylation in C-starved cells becomes elevated when respiratory activity drops to very low levels, PAPER I (10). From these results it can be concluded that high respiratory activity does not necessarily coincide with high levels of protein carbonylation in *E. coli*.

In yeast, increased carbonylation was linked to an increased propensity of the mitochondria to produce ROS (4, 78), but, similar to *E. coli*, not to increased respiratory rate. Starvation for either carbon or nitrogen caused a drastic drop in respiratory rate, but also elicited a so-called respiratory shift from a 'state 3'-type respiration to a 'state 4'-type. This shift uncouples electron transport in the respiratory chain and ATP-production and leads to increased membrane potential, high ROS production and elevated protein carbonylation. It is not known weather such a respiratory shift occurs also in starved *E. coli*. At any

rate, these results argue against the 'rate-of-living' hypothesis, but not the free radical hypothesis of ageing, since low oxidation damage correlates with long lifespan/starvation survival.

Work on the *E. coli* model system also demonstrates that increased production of ROS is not a prerequisite for increased protein carbonylation. Instead, carbonylation can occur as a direct consequence of proteins becoming more susceptible to oxidative attack. Aberrant proteins are such targets (42). Analyses in *E. coli* have demonstrated that this pathway for carbonylation is more general i.e. targets a broader spectrum of proteins than the specific ageing/senescence-associated carbonylation and highlights a link between translational accuracy and protein oxidation as described below, PAPER I, PAPER IV (10, 54, 138).

Upon starvation-induced growth arrest, mistranslation, i.e. erroneous incorporation of amino acids in the polypeptide chain increases, PAPER I (10, 12, 145). The most simple explanation for this is a starvation-dependent, reduced availability of charged tRNAs, reflecting a change of cellular metabolism, rather than an intrinsic change in ribosomal accuracy, PAPER I (10, 12, 142, 196).

Starvation-dependent mistranslation results in many proteins being produced in different isoforms, which can be detected on 2D-gels. The different isoforms migrate differently from the authentic proteins during the isoelectric focusing. This phenomenon is referred to as 'protein stuttering' and is strongly associated with increased carbonylation of the mistranslated proteins, PAPER I, PAPER IV (10, 54, 145). Starvation-induced mistranslation, protein stuttering and protein carbonylation can all be alleviated by a mutation in the ribosomal protein S12 (the *rpsL141* allele) that renders the ribosome hyper-accurate and less error prone, PAPER I (10). On the other hand, cells harbouring sloppy ribosomes caused by the *rpsD12* allele of the ribosomal protein S4, shows increased mistranslation, protein stuttering and carbonylation, PAPER I, PAPER

IV (10, 54). Since the ribosomal accuracy mutations do not affect respiratory activity or the oxidant defence (Sod activity), PAPER I (10, 42), these experiments demonstrate that increased protein carbonylation does not, *per se*, require elevated production of ROS. Instead increased availability of targets for ROS (aberrant proteins) seems to be the key determinant for, at least, starvation-induced general protein carbonylation in the early stage of *E. coli* stationary phase, PAPER I (10, 42). The demonstration that aberrant proteins have elevated sensitivity to oxidative attacks resulting in carbonylation, is further supported by the fact that protein carbonylation is elevated by drugs such as puromycin and streptomycin that cause translational errors (42).

It is not yet clear why aberrant proteins are more susceptible to carbonylation than native proteins, but possibly, misfolding of a protein exposes oxidation-sensitive amino acid residues normally buried in protein structure during the coupled translation folding process (69).

Protein carbonylation – a general or selective event?

The carbonylation that occurs in growth arrested *E. coli* cells subsequent to the initial burst of oxidation linked to mistranlation, is more 'specific' and targets fewer protein species. These targeted proteins are involved in a variety of functions such as stress protection, protein quality control, information transfer, energy transfer and metabolism, genome organisation and other, PAPER II (39, 43, 44, 53, 184). Among target proteins are DnaK and GröEL, elongation factors, EF-Tu and EF-G, the histone-like protein, H-NS, aconitase, glutamine synthetase, glutamate synthase, pyruvate kinase, pyruvate dehydrogenase (E2 subunit) and TCA-cycle enzymes like malate dehydrogenase, PAPER II (43, 53, 184). Several of these proteins have similarly been found to be carbonylated in oxidation stressed yeast (23); ageing flies (174, 200), plants (89, 102), and in human Alzheimer's disease brain (25). Considering the distant relationship

between these organisms, carbonylation sensitivity of proteins seems to be at least to some extent, evolutionary conserved.

Although it is clear that proteins have different intrinsic sensitivity to carbonylation, the molecular basis for this selectivity is not fully understood. However, there are some factors that have proven to be involved. For example, it is likely that metal catalyzed oxidation, e.g. the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$) (180) is a built-in problem for proteins containing transition metals, e.g. aconitase. However, some but not all of the identified carbonylated proteins are known to bind metals.

The chemical composition of the polypeptide chain may be important, since carbonylation occurs preferentially on lysines, arginines, prolines and threonines as described. Proteins rich in these amino acids may have a high susceptibility to carbonylation. One such example may be the mitochondrial adenine nucleotide translocase (ANT) that becomes carbonylated in for example ageing house flies (201). ANT contains a relatively large number of lysines, arginines and prolines (201).

The fact that misfolded and aberrant proteins are intrinsically sensitive to carbonylation indicates that the structure and conformation of a protein could affect oxidant susceptibility also of native proteins. It is possible that some proteins have a structure that is more prone to become oxidized, e.g. by having an intrinsically unstable composition or a tendency to change conformation, for example upon substrate release. Glutamine synthetase may be an example of the latter. It becomes oxidatively modified and subsequently degraded upon starvation for its substrate (32, 112, 160, 163) (see section 'Protein oxidation – Damaging protein oxidation – carbonylation'). Enzymes are frequently found to be protected from degradation while bound to their substrates (123), but whether this also confers protection against oxidation remains to be elucidated.

Another idea holds that proteins, e.g. enzymes of the TCA-cycle and electron transport chain, become oxidized simply because of their location in

close proximity of ROS generating sites (138). All these mechanisms are suggested to be implicated in senescence-related carbonylation of proteins, but the relative importance of these individual mechanisms remains to be elucidated.

Removal and repair

Degradation of oxidized proteins seems to be a universal and conserved defence mechanism against senescence-related cumulative oxidative protein damage among different cell types, and therefore reduced proteolytic capacity may be implicated in age/senescence-related accumulation of carbonylated proteins (56, 121, 138, 172).

In mammalian cells, proteasomes and lysosomes are two of the major proteolytic systems. Loss/decline of function upon ageing and senescence has been reported for both (28, 122) and this correlates with the accumulation of damaged e.g. carbonylated and aggregated proteins. Interestingly, such proteins has, together with other oxidation derived aggregates e.g. lipofuscin/ceroids, been suggested to eventually clog up the proteasome and thereby be a cause of age-dependent reduced proteolysis (28, 138). In addition, direct oxidative damage and other modifications of the proteasome, as well as an unbalanced expression of the proteasome subunits, may also cause age-related proteasome dysfunction (29). Interestingly, partial inhibition of the proteasome in young primary fibroblasts induced a premature ageing phenotype including accumulation of damaged proteins (29), while overproduction of proteasome β_5 assembled subunit increased the amount of proteasome and conferred an ameliorated response to oxidative stress and higher survival rates (30)

Another example that links protein oxidation and lifespan to proteolysis concerns the age-dependent carbonylation of aconitase in mitochondria. Decreased aconitase activity caused by carbonylation has been associated with shortened lifespan and senescence and has been linked to an age-dependent decline in the levels and/or activity of the mitochondrial Lon protease (19, 20,

37). Lon degrades moderately oxidized aconitase, while more severely oxidized forms thwarts the proteolytic apparatus and aggregate in electron-dense inclusion bodies within the mitochondrial matrix (17, 19). In addition to the decreased aconitase activity that may be detrimental itself as discussed in section 'Possible roles of carbonylation – some speculations – Regulation of specific pathways and enzyme function', such aggregation may eventually lead to mitochondrial collapse, elevated ROS production and increased oxidative stress.

Also in growth arrested *E. coli*, the Hsp protease Lon is required to prevent accumulation of carbonylated proteins. Deletion of *lon* strongly increased the accumulation of carbonylated proteins in growth arrested cells and deletion of *hslVU*, encoding the Hsp protease HslVU, had an even more pronounced effect, PAPER II (53). This increased carbonylation of proteins were, for the most part, non-specific in the sense that the same spectrum of oxidized proteins exhibited a higher load of carbonyls in both mutants, PAPER II (53). Since Lon is the primary protease degrading misfolded and aberrant proteins in the *E. coli* cytosol (161, 190), Lon may prevent accumulation of carbonylated proteins via at least two pathways, including direct degradation of carbonylated proteins and/or by preventing the formation of carbonylated proteins by proteolytic degradation of aberrant proteins produced via mechanisms not linked to oxidation.

It has been argued that oxidative modification of a protein makes it more susceptible to proteolysis via mechanisms including unfolding of the polypeptide chain and exposure of hydrophobic patches normally hidden in protein structure (69, 138). Such hydrophobic patches favour recognition and degradation by for example the proteasome and the Lon protease (69, 70). This process may be enhanced by chaperone activities.

In addition to proteolysis, protein repair systems may be implicated in preventing carbonylation of proteins. Once formed, protein carbonyls, similar to

most other types of oxidative protein damage, are irreparable. However, methionine sulfoxide, cysteine disulfides, and mixed disulfides are reversible types of oxidative damage which can be readily reduced back to the corresponding thiols by a battery of reductases and isomerases (36). Repair of such damage may decelerate carbonylation, by preventing transfer of oxidative damage within proteins, and also, if the oxidation induced a conformational change, by restoring protein structure.

Molecular chaperones, of which many are Hsps (or Hscs) constitute powerful protein repair and protection systems. Chaperones participate in a plethora of protein management processes related to e.g. protein production, refolding, remodelling, and proteolysis. The chaperones (e.g. DnaK) identify substrate proteins, both by recognition of specific sequences on proteins, and by their affinity for hydrophobic domains (e.g. on misfolded proteins) (47, 52). Similar to proteolysis, the latter process may be enhanced by oxidation of the misfolded protein as indicated in, PAPER II (53).

Overprodution of DnaK and its co-chaperone DnaJ in growth arrested *E. coli*, was demonstrated to strongly reduce protein carbonylation, despite the fact that such overproduction down-regulates the expression of all other Hsps, PAPER II (53). The underlying mechanism for the decreased carbonylation is not clear, but the DnaK/J system is not expected to repair or refold carbonylated proteins since the modification is irreversible. Instead, it may reduce the abundance of aberrant proteins available for oxidative attack and/or keep oxidized peptides and proteins in a soluble, protease-accessible state.

How does DnaK discriminate between delivering a substrate protein to a refolding or proteolytic pathway? Maybe this process is simply stochastic and time-dependent. For example, carbonylation alters the chemical properties of amino acids. This may render proper refolding impossible and the substrate protein will thus remain in a partly misfolded state. Binding of DnaK may prevent aggregation of the polypeptide chain with other unfolded or damaged

proteins, and may also increase its accessibility to proteolysis via DnaK-mediated unfolding. With time the likelihood of bumping into a protease by the chaperone/substrate-complex increases leading to degradation of the misfolded protein. By such a mechanism, carbonylation of a protein may ensure it is properly degraded.

Taken together, protein management functions seem to be essential in the cellular protection against cumulative protein damage. Recent experiments in *E. coli* and other organisms provide data where the Hsps emerge as key players in protection against senescence-related protein oxidation PAPER II (53, 115, 129, 131, 204).

Possible roles of carbonylation - some speculations

Evolutionary considerations

At first glance, carbonylation of proteins may appear devastating for cells, tissues and the whole organism. Carbonylation induces enzymatic dysfunction, severe structural aberrancies, aggregation of proteins into proteolysis resistant complexes with strong cytotoxic effects and is strongly associated with disease and senescence. Therefore, an interesting issue is why natural selection has not been able to obliterate this process.

For an organism that depends on aerobic metabolism, it may of course be an impossible task to fully combat oxidative damage of cellular components since oxygen is ubiquitously present. However, as demonstrated in this work, it is possible to drastically reduce protein carbonylation experimentally, e.g. by ectopic overproduction of the Hsps, PAPER II (53), by genetically increasing the accuracy of the ribosomes, PAPER I, PAPER IV (10, 54) and by other pathways as well (43).

So why are not the defence systems against this deleterious modification of proteins at their maximum by nature? Possible explanations may be provided by the 'disposable soma' and 'antagonistic pleiotrophy' theories of ageing. The former suggest that the resources an individual can allocate to its activities is limiting such that growth/reproduction and maintenance are traded, one at the expense of the other (99, 100). Rapidly dividing cells may escape part of the 'resource-cost' related to intracellular damage, simply by 'dilution' of the injured molecules by *de novo* synthesis and cell division; a possibility not available for post-mitotic cells.

In nature, organisms do not usually die of old age, but are eaten, parasitized, or out-competed by others. The idea of antagonistic pleiotrophy holds that genes which' expression are beneficial during development and reproduction early in life, might have detrimental consequences later on (15,

100). Since a post-reproductive organism is under low (if any) evolutionary pressure, there is no strong selection against deleterious processes such as protein carbonylation late in life as long as it does not affect fitness (80). In addition, if cells/organisms can take advantage of carbonylation of proteins whilst still in the reproductive phase, evolution will not work against this mechanism, and carbonylation will occur also during non-reproductive stages despite the fact that it may have negative effects on the soma/individual. Such an advantageous role of protein carbonylation may be in protein quality control.

Protein quality control

Mistranslated and otherwise aberrant proteins are more sensitive to carbonylation than native proteins, PAPER I, PAPER IV (10, 42, 54) and carbonylated proteins are more susceptible to proteolytic degradation than their non-oxidized counterpart (17, 20, 42, 69, 70, 163). Carbonylation of mistranslated proteins could thus be a mechanism to avoid incorporation of injured proteins into cellular machines involved in information transfer (e.g. ribosomes and RNA and DNA-polymerases) (138). In line with this argument, the levels of carbonylated ribosomal proteins are relatively low in healthy, starving *E. coli* cells (39), but is strongly elevated in cells that have lost their reproductive capacity (see section 'Protein oxidation – Protein carbonylation and ageing'). The high load of carbonylation of ribosomal proteins in these sterile cells may result in ribosomal dysfunction. This could imply that impaired protein synthesis, at least partly, causes the sterility and subsequent death of this subpopulation of stationary phase *E. coli* cells. The accumulation of carbonylated ribosomal proteins may be a consequence of some sort of dysfunction in the proteolytic capacity of these cells and one candidate protease that could be involved is Lon. In *E. coli*, Lon has been demonstrated to degrade ribosomal proteins after a nutritional down-shift (amino acid starvation) (105) and deletion of *lon* results in extensive accumulation of carbonylated proteins

and also reduced survival, PAPER II (53), non-published data, this work). It is possible that if Lon-functions are abated, this reduces starvation-induced degradation of ribosomal proteins, leading to their time-dependent carbonylation, and consequentially an error-catastrophe-feed-back loop as suggested by Orgel (144). Thus it is possible that carbonylation tags the protein for degradation and that this functions as a protein quality control.

In eukaryotes, proteins are labelled for degradation by the proteasome by enzymatic addition of tails of the protein ubiquitin: a process referred to as ubiquitinylation (8). Whether carbonylation and ubiquitinylation work in concert or are completely separate processes for tagging a protein for proteolytic degradation is not clear (138), but carbonylated proteins can be recognized and degraded by the mammalian 20S proteasome independently of the ubiquitinylation system as well as in the absence of ATP (35).

Protein carbonylation and reproduction

If carbonylation is entirely bad, there may still be a threshold level which can be tolerated before systemic collapse and reproductive failure commences. Therefore, an investment in improved defence against carbonylation only maximizes fitness if the resources are not better invested in improving other capacities important for reproduction, e.g. speed, intelligence, eye-sight, beauty, etc.

An interesting study in the plant *A. thaliana* demonstrated that protein carbonylation can be tolerated during pre-reproductive adulthood (89). In the first 20 days of the life cycle, carbonylation increased with time, but prior to bolting and flower development (89) there was a drastic reduction in protein carbonyls. It would be interesting to determine whether such reduction in protein carbonyls is a prerequisite for the production of reproductive organs and if the fitness of the offspring is ensured by this mechanism. Another example of keeping the offspring free of carbonylated proteins is seen in the yeast

Saccharomyces cerevisiae, which display asymmetrical cytokinesis. During such cytokinesis, the older mother cell retains the carbonylated proteins, a process that may ensure a full replicative life span of the daughter and/or its fitness (3).

Together, these studies points to a mechanism that may serve to protect the offspring from inheriting carbonylation damage from the parent, and the data may be interpreted such that protein carbonylation, at least in yeast and *A. thaliana* have negative effects on early fitness. These results do not discriminate between the two possible interpretations, 1) that carbonylation of proteins is simply a negative consequence of the events and activities necessary for reproduction or 2) if there are, speculatively, beneficial roles of protein carbonylation during pre-reproductive adulthood.

Carbonylation and autophagy-like mechanisms

In *E. coli, de novo* protein synthesis is an absolute requirement for survival of growth arrest and starvation. Degradation of proteins that are either damaged or simply not longer needed may provide an internal source of amino acids for this translation, as described above.

It is possible that carbonylation enhances such an autophagy-like mechanism by labelling of dispensable proteins and, due to the protein destabilizing effect of carbonylation (42), accelerates the proteolytic efficiency. In line with this, there is a burst in protein carbonylation early upon starvation, PAPER II (53) that targets a broad spectrum of proteins of which many are metabolically dispensible. As described in the section 'Protein oxidation – Damaging protein oxidation – carbonylation', glutamine synthetase is carbonylated and subsequently degraded upon nitrogen starvation (110). It is possible that this apply also to other enzymes.

Not only may carbonylation tag proteins for degradation during adaptation of *E. coli* to growth arrest. Also, resumption of growth may be

facilitated if an excess of energy/resource-consuming stress-proteins no longer needed are rapidly degraded. In line with this, Hsps, e.g. DnaK, are sensitive to carbonylation, PAPER IV (43, 54). However this reasoning leaves us with the question of how carbonylation can be selective (see section 'Protein oxidation – Protein carbonylation – a general or selective event?').

It is possible that evolution has selected for protein structures and properties that affect their intrinsic susceptibility to carbonylation. If so, this suggests that cells may to some extent, 'direct' carbonylation and take advantage of such modification as an additional regulatory mechanism of protein stability.

Regulation of specific pathways and enzyme function

Most enzymes do not lose their catalytic functions during senescence (174). However, among those that do, there seems to be some conserved, interspecies target specificity of ageing/senescence related carbonylation, suggesting that carbonylation is not a random process. Among such targets are the TCA-cycle enzyme aconitase and the chaperone Hsp70, PAPER IV (20, 32, 54, 89, 200, 201). These proteins are used as examples in the discussion below on possible benefits and drawbacks of specific senescence-related carbonylation.

In eukaryotes carbonylation of mitochondrial aconitase has been suggested to trigger ageing-related, deteriorative processes. Aconitase loses its catalytic activity when carbonylated. This may cause strong imbalance in the TCA-cycle (58) and accumulation of TCA-cycle intermediates that contributes to a decline in the overall efficiency of mitochondrial bioenergetics and diverts intermediates to other pathways. For example, citric acid and succinate have been identified as ligands for G-protein coupled receptors and the release of these TCA-cycle intermediates from mitochondria was suggested to provide a link between protein-specific carbonylation and age-related diseases such as diabetes, atherosclerosis and hypertension (73, 74, 203).

Carbonylation may also serve to immediately destroy a specific enzymatic activity to ensure a fast shut off of pathways that may otherwise cause damage during a certain condition. Results from studies in *E. coli* may be considered in this context. Several TCA-cycle enzymes become carbonylated upon aerobic starvation of *E. coli*, PAPER II (43, 44, 53, 184). Such damage of these enzymes may contribute to the metabolic swap (including strongly decreased respiratory activity) that occurs upon carbon-starvation, preceding the pathways down-regulating TCA-cycle gene expression (10, 137, 140) (section 'The experimental system – Physiological alterations in stationary phase – an oxidative stress defence?'). This may secure a fast rearrangement of cellular metabolism in response to starvation.

On the other hand, carbonylation of Hsp70 proteins during stress conditions may seem obscure considering its cyto-protective role in general and against cumulative protein carbonylation in particular, PAPER II (53). Carbonylation of Hsp70s may be accidental and detrimental, but there may as well be positive effects of such damage as implied in, PAPER IV (54), since Hsp70-proteins are part of the regulatory systems controlling the overall Hsp-production. For example, in *E. coli*, oxidatively damaged DnaK may be unable to recognize and deliver σ^{32} to proteolysis (see section 'The σ^{32} regulon'). This may speed up or boost the induction of the heat shock proteins through a mechanism independent of increased σ^{32}-synthesis, but instead related to its' rapidly increased stability, PAPER IV (54). A similar positive effect may occur in eukaryotes upon carbonylation of Hsp70, since Hsp70 is a negative regulator of HSF activity. Thus, Hsp70 proteins may have a function as sensors of cellular oxidation status and the suggested mechanism for this would result in rapid adjustments of Hsp production PAPER IV (54). In addition, since in *E. coli*, both σ^{32} and σ^{S} are regulated at the level of stability, titration of their proteases by carbonylated (and other oxidized) proteins confers another, although indirect regulatory function of protein carbonylation, PAPER V (54).

Thus, by a process of conclusion, we arrive at the fact that protein carbonylation may be both a blessing and a menace. While it is obvious that carbonylation of specific enzymes severely affects metabolic and other pathways in cells and that this may exacerbate deteriorative processes, it is also possible that cells to some extent may take advantage of carbonylation in, for example, protein quality control and in regulatory functions. Further, the Hsps seems to be key players in the management of carbonylated proteins and have strong cyto-protective functions against its negative effects.

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