Section IV

Oxidative Folding and Cellular/Organism Homeostasis

CHAPTER 4.1

How Microbes Cope with Oxidative Stress

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4.1.1 Reactive Oxygen Species – an Inevitable Consequence of Aerobic Life

AQ1 The presence of oxygen in the atmosphere promotes optimal energy production and supports the rapid growth of microorganisms. However, living in a world with oxygen comes with a heavy burden. Reactive oxygen species (ROS), particularly hydrogen peroxide (H_2O_2), superoxide (O_2^{-}) and hydroxyl radicals ($HO^{\cdot -}$), are continuously generated as by-products of aerobic respiration and by enzymatic processes involving NAPDH oxidases or cytochrome P450.[1,2] Although necessary in small amounts for cellular signaling purposes,[3] excess levels of ROS can cause devastating cellular effects, ranging from DNA damage and fatty acid oxidation to the modification of amino acid side-chains and cofactors in proteins. If produced uncontrolled and not detoxified properly, ROS will accumulate and elicit a stress response generally termed oxidative stress.[4]

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Microorganisms have developed a complex network of enzymes and small molecules to prevent the accumulation of ROS and maintain the redox homeostasis of the cell.[5] The thioredoxin system, for instance, is dedicated to reducing oxidative thiol modifications in cytosolic proteins and provides electrons for more than 250 substrates in *Escherichia coli*.[6] The glutaredoxin system also reduces oxidative thiol modifications and is responsible for upholding a high ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG), thereby maintaining a reducing redox potential in the cell (for reviews, see ref. 7 and 8). Both glutaredoxin and thioredoxin systems draw their reducing powers from NADPH, which is supplied primarily by the pentose phosphate pathway. In addition, organisms have evolved an arsenal of detoxifying enzymes to prevent ROS accumulation. These include superoxide dismutase (SOD), which converts superoxide into H_2O_2 and O_2, and also peroxidases and catalases, which detoxify H_2O_2 into harmless water.[2,9]

4.1.2 Oxidative Stress – an Effective Mammalian Host Defense Mechanism

Owing to the unlimited supply and the potentially highly toxic effects of ROS, it is not surprising that some organisms use localized oxidant production as a powerful antimicrobial defense strategy.[10] One such example is the innate immune system in mammals. Once activated, macrophages and neutrophils not only copiously produce and secrete peroxide but also convert peroxide into various hypohalous acids (HOX), including the reactive chlorine species (RCS) hypochlorous acid (HOCl, bleach), hypobromous acid (HOBr) and hypothiocyanous acid (HOSCN), to kill invading pathogens and control bacterial colonization.[11–16] Produced by a variety of different peroxidases, including neutrophilic myeloperoxidase and eosinophil peroxidase, hypohalous acids are found at high concentration at sites of inflammation and also in saliva and at mucosal barrier epithelia.[17–19] All three hypohalous acids are significantly more antimicrobial than H_2O_2.[19] Although HOCl and HOBr appear to be equally toxic to both microbes and mammals, HOSCN is much better tolerated by mammalian cells, which can detoxify HOSCN through their selenium-containing thioredoxin reductase.[18,20] In summary, production of hypohalous acids can be considered as the true first line in mammalian host defense, limiting bacterial colonization, mitigating pathogenicity and hence combating infections.[14,17,21,22]

4.1.3 Cellular Effects of Antimicrobial Oxidants

Individual ROS, RCS and other antimicrobial oxidants differ significantly in their specific biological properties, making their combined production an even more powerful mechanism to cause damage.[2,23,24] Superoxide and hydrogen peroxide, for instance, preferentially oxidize (and destroy) iron–sulfur clusters and mononuclear iron-associated proteins.[25–27] Hydroxyl

radicals, which are produced when peroxide reacts with Fenton metals, such as the iron released from iron–sulfur clusters, are extremely reactive and non-specifically oxidize most biomolecules, mainly DNA, lipids and proteins.[28] *In vivo*, $HO^{\cdot}$ favors DNA since part of the cellular iron pool is closely associated with DNA. In the case of hypohalous acids, HOCl, best known as the active ingredient of household bleach, causes extensive protein unfolding and aggregation, presumably by oxidizing amino acid side-chains, especially those of cysteines, methionines and histidines.[24,29–31] HOCl and HOBr also interact with fatty acids and cholesterol, causing extensive membrane perturbation,[13] whereas HOSCN is thought to oxidize primarily thiols, tryptophans and selenium cofactors.[32] Recent comparative RNA_{seq} and proteomics analyses revealed that HOCl and HOBr affect similar cellular targets, and elicit overlapping transcriptional responses that are consistent with widespread oxidative protein unfolding and aggregation.[33] HOSCN seems to affect membrane proteins and also various metabolic enzymes, especially glycolytic enzymes. This explains how HOSCN effectively perturbs cellular energy production,[22] and agrees with the observation that HOSCN, in contrast to HOCl and HOBr, is especially effective in killing actively growing bacteria.[33]

4.1.4 How Bacteria Cope with Antimicrobial Oxidants

Oxidative insults trigger both specific and global cellular responses involving transcriptional, post-translational and metabolic adaptations. In this section, we provide an overview of all of these aspects, but focusing primarily on the most recent discoveries in the sensing of and response to antimicrobial oxidants, particularly its most powerful member, the reactive chlorine species (RCS) hypochlorous acid (HOCl, bleach).

4.1.4.1 Transcriptional Changes in Response to ROS and RCS

A rapid response to environmental changes is key for organisms to adapt to and survive stress. To sense ROS and RCS, such as HOCl, bacteria use primarily post-translational modifications that are directly triggered by the respective oxidants, and hence provide the necessary specificity in the response. The two best studied (and most reviewed) transcriptional regulators in bacteria are OxyR and SoxR. OxyR, which is a LysR family transcriptional activator, specifically senses peroxide using reversible disulfide bond formation as the activation mechanism.[34,35] SoxR, which is a MerR family transcriptional activator, senses superoxide through oxidation of an iron–sulfur cluster.[36,37] Both transcriptional regulators, once oxidatively modified, alter their DNA binding affinity, causing the increased expression of genes, whose products detoxify the specific oxidants, repair the oxidative damage and restore redox homeostasis.[38] In addition to these two transcription regulators, however,

a number of novel oxidative stress-responsive regulators have emerged in recent years. Here we focus primarily on newly discovered RCS-sensing transcriptional regulators in *E. coli*, which, in our opinion, beautifully illustrate the existing diversity in oxidative stress sensing and responses.

*4.1.4.1.1 The HOCl–*N*-Chlorotaurine Response System RclR*

The "reactive chlorine resistance" (*rcl*) genes *A*, *B* and *C*, together with their transcriptional regulator RclR, a member of the highly conserved AraC family, have been identified as the most highly upregulated genes in *E. coli* in response to sublethal HOCl treatment (Figure 4.1.1a).[39] Deletion of any one of the four genes causes increased sensitivity towards RCS such as bleach or

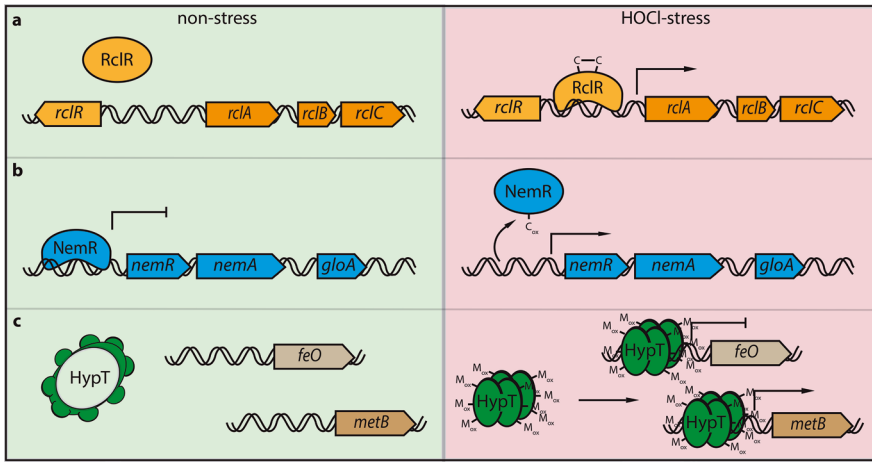

Figure 4.1.1 HOCl-dependent transcriptional regulators in *E. coli*. *E. coli* encodes at least different transcriptional ems that respond to HOCl treatment. Each regulator senses I and related RCS by a specific mechanism and responds with the upregulation of a specific subset of genes. (a) RclR acts as a transcriptional activator. HOCl-dependent oxidation of two key cysteines causes the formation of an intramolecular disulfide bond in RclR, which stimulates RclR DNA-binding activity and leads to the expression of the *rcl* operon. (b) NemR acts as a transcriptional repressor. Under non-stress conditions, NemR is tightly bound to its recognition sequence, repressing the *nemRA* operon. HOCl-dependent oxidation of a key cysteine to sulfenamide causes the release of NemR and leads to the expression of glyoxylase (gloA), a methylglyoxal detoxifying enzyme, and the reductase NemA. (c) HypT is a transcriptional activator. Under non-stress conditions, HypR adopts an inactive dodecameric ring-like structure. HOCl-mediated oxidation of three methionine residues leads to the dissociation of the HypT ring into transcriptional active tetramers, which bind DNA at specific promoter sites, upregulating genes involved in methionine and cysteine biosynthesis (*e.g. metB*) and downregulating genes involved in iron uptake (*e.g. feO*). Green indicates non-stress conditions and red indicates HOCl treatment.

N-chlorotaurine, a secondary oxidation product of HOCl *in vivo*.[39,40] Deletion of RclR abolishes the induction of *rcl* genes upon RCS treatment, confirming that RclR functions as a transcriptional activator.

Mechanistic studies revealed that HOCl leads to the reversible formation of an intramolecular disulfide bond in RclR (Figure 4.1.1a).[39] Mutant variants of RclR lacking either one of the two cysteines lose their ability to respond to HOCl treatment *in vivo*. These results are highly reminiscent of the activation mechanism of OxyR, which also contains two cysteines that form one activating, intramolecular disulfide bond upon peroxide treatment.[35,41] In contrast to OxyR, however, RclR is highly RCS specific and unreactive towards ROS such as H_2O_2.[39] These results agree with the notion that peroxide exerts generally a very low reactivity towards cysteine residues (10 L mol^{-1} s^{-1}), and oxidizes only extremely reactive thiol groups, such as present in OxyR or peroxiredoxin.[42] HOCl, on the other hand, has reaction rates that are up to seven orders of magnitude higher,[30] causing rapid (and often indiscriminate) chlorination and subsequent oxidation of thiol groups.[43] Oxidation of RclR appears to be fully reversible both *in vitro* and *in vivo*, hence guaranteeing that the response is terminated once reducing conditions have been restored.[39] Neither the structural changes in RclR that lead to the induction of gene expression nor the role and contribution of each of the genes within the *rill* region towards bleach resistance are known.

4.1.4.1.2 The HOCl–Methylglyoxal Response System NemR

NemR, a member of the TetR family of transcriptional repressors, controls the expression of the *nemRA* operon. This operon encodes *nemR* itself, the glyoxylase *gloA* and a predicted reductase *nemA*.[44] Under non-stress conditions, binding of NemR to a palindromic sequence upstream of the *nemRA* operon prevents transcription (Figure 4.1.1b).[44] Induction of the NemR regulon appears to be specific to either electrophiles, such as *N*-ethylmaleimide (NEM) and methylglyoxal (MGO),[44,45] or RCS, such as HOCl.[46] *In vitro* binding and mechanistic studies suggested that NemR senses HOCl through the reversible oxidation of a single, highly conserved cysteine residue to sulfenamide.[47] This oxidation leads to the dissociation of NemR from the DNA and, in turn, induces *gloA* and *nemA* expression (Figure 4.1.1b).[46,48] Surprisingly, deletion of the *gloA* gene, which encodes the MGO-detoxifying enzyme glyoxylase,[49] had the most severe effect on HOCl survival, suggesting a previously unrecognized connection between MGO production and bleach stress.[46]

MGO is a powerful electrophile, capable of forming advanced glycation end products in both proteins and nucleic acids, particularly guanine bases.[50,51] Formed *in vivo* from the glycolytic intermediate dihydroxyacetone phosphate (DHAP).[52] MGO accumulation occurs naturally in *E. coli* under simultaneously low intracellular phosphate and high DHAP levels. These conditions arise, for instance, when cells are shifted from starvation medium to rich medium, and serve to replenish cellular phosphate stores.[53] Our transcriptional

studies revealed that HOCl treatment leads to severe phosphate starvation. This observation, combined with the known HOCl-mediated inactivation of glyceraldehyde-3-phosphate dehydrogenase, which causes DHAP accumulation, explains why HOCl treatment leads to toxic MGO production in *E. coli*.[46] It also explains why an HOCl-induced transcriptional regulator (*i.e.* NemR) induces a glyoxylase, whose primary function is to convert MGO into harmless lactate.[49] The role of NemA, a flavin-dependent reductase, is less clear. *In vivo*, NemA appears to be involved in reducing quinones to quinols, which might help to restore the redox balance upon stress.[48]

4.1.4.1.3 The HOCl Response System HypT

HypT is the third redox-regulated transcriptional factor that appears to contribute specifically to increasing HOCl resistance in *E. coli*. Deletion of *hypT* leads to increased bleach sensitivity but shows no effect on other ROS resistances.[54] Transcriptional studies revealed that HypT, a LysR family member, increases expression of genes involved in methionine, cysteine and sulfur metabolism while decreasing expression of genes involved in iron uptake and homeostasis.[54] Under non-stress conditions, HypT forms dodecameric ring-like oligomers (Figure 4.1.1c).[54] Oxidation of three key methionine residues to methionine sulfoxide appears to trigger the conversion of inactive HypT dodecamers into highly active DNA-binding dimers and tetramers (Figure 4.1.1c).[55,56] Given that the reactivity of HOCl towards methionines is up to nine orders of magnitude higher than that of H_2O_2,[57] using methionine oxidation as an activating mechanism provides an effective basis for HOCl specificity. Oxidation and activation of HypT are reversed by methionine sulfoxide reductases MsrA/B,[55,56] which are dedicated enzymes that restore the cellular methionine oxidation state.[58]

4.1.4.1.4 Other Bacterial Redox-sensitive Transcriptional Regulators

We would not do the growing redox field justice if we did not draw the reader's attention to several other redox-sensitive transcription factors that have recently been discovered in bacteria such as *Bacillus subtilis*, *Streptomyces coelicolor*, *Mycobacterium tuberculosis* and *Firmicutes* (for a review, see ref. 59). These include MarR-type regulators, such as OhrR, which senses organic hydroperoxides *via* reversible sulfenic acid formation,[60] anti-sigma factors, including RsrA, RseA and RshA, which sense peroxides and/or HOCl,[61,62] and Fur-type regulators such as PerR and others that are activated by H_2O_2,[38,63] All of these proteins contain either redox-sensitive cysteines (OxyR, SoxR, OhrR, RsrA and RshA) or, more rarely, histidine residues (PerR) at strategically important sites. Once oxidized by their respective ROS or RCS, these modifications trigger conformational changes in the respective proteins, which then induce highly specific transcriptional responses. The expression changes affect primarily genes involved in

(i) detoxification of oxidants in order to restore the redox balance (*e.g.* glutaredoxin, SOD), (ii) DNA repair mechanisms and chaperones to repair and prevent oxidative damage[31,54] or (iii) metabolic enzymes to provide the necessary cellular resources (*e.g.* NADPH) to restore redox homeostasis.[64] By using an arsenal of ROS/RCS-specific transcriptional regulators, microorganisms have therefore developed effective and powerful strategies to evade the mammalian host defense, making combating infections without antibiotic therapies more challenging.

4.1.4.2 Metabolic Changes Upon ROS–Antimicrobial Oxidant Insults

Exposure of cells to high levels of ROS and RCS leads to a global reorganization of cellular metabolism. One of the most striking events is a rapid and dramatic decrease in intracellular ATP levels. This event, which occurs in response to ROS and RCS in both pro- and eukaryotes, has long been attributed to the oxidative inactivation of glycolytic enzymes, such as GAPDH, and other enzymes of the central carbon metabolism.[65–68] Cessation of glycolysis at the step of GAPDH effectively reroutes glucose towards the pentose phosphate pathway, which generates NADPH used in ROS detoxifying and redox-restoring reactions.[69] In addition, however, evidence from our laboratory suggested that in bacteria, active conversion of ATP into long chains of polyphosphate also contributes significantly to the observed ATP decline upon HOCl stress (see below for more details).[70] Another metabolic event that occurs during oxidative stress is the upregulation of the glyoxylate shunt, which bypasses three of the eight reactions of the Krebs cycle, thereby reducing the production of NADH and carbon dioxide.[71,72] In doing so, cells decrease the flux of electrons funneled into respiration. This reduces additional ROS production while preserving carbon atoms.[73] At the same time, cells upregulate the biosynthesis of methionine and cysteine to replenish those amino acids that are the primary targets of oxidative modifications.[54,74] Finally, transcription of genes involved in aerobic metabolism is repressed whereas expression of ROS-resistant isoforms of metabolic enzymes such as fumarate hydratase and aconitase A is induced.[75,76] These results illustrate the strategies that bacteria developed to adjust their metabolism rapidly to survive oxidative stress conditions, including halting ATP production to store energy, avoiding the generation of additional ROS and increasing the reductive power for detoxification.

4.1.4.3 ROS-mediated Activation of ATP-independent Molecular Chaperones

Stalling ATP synthesis and converting ATP into isoenergetic polyP serves as a clever strategy to preserve energy resources while downregulating oxidative stress-sensitive processes.[70] The drawback, however, is that many

proteostasis factors rely on ATP as an energy source.[77] Reduction in cellular ATP levels becomes especially critical for canonical folding chaperones such as bacterial GroEL and DnaK, which assist in the correct folding of nascent and the refolding of unfolded polypeptides through ATP binding and hydrolysis.[78,79] In addition, some chaperones are themselves oxidatively modified by ROS or RCS, further compromising the chaperoning capacity of the cell.[80,81] To deal with protein unfolding and aggregation under oxidative stress conditions, bacteria have therefore evolved specialized chaperone systems that rapidly become post-translationally activated by ROS or RCS, work ATP independently, and whose primary function is not to fold proteins but simply to prevent their irreversible, often toxic, aggregation.[82] In the following we review two redox-regulated chaperone systems that contribute to the increased ROS/RCS resistance in bacteria. Each of them exerts its own ROS/RCS-specific activation mechanism and mode of chaperone function.

4.1.4.3.1 Hsp33 – Activation by Reversible Disulfide Bond Formation

One of the first cytosolic proteins that were described to gain function upon oxidative disulfide bond formation was the 33 kDa heat-shock protein Hsp33, which turns into an effective chaperone only upon exposure to oxidative stress conditions (Figure 4.1.2).[83] Under non-stress conditions, Hsp33 is inactive and contains one zinc(II) ion coordinated by four absolutely conserved cysteines located in the C-terminal redox switch domain.[83] Incubation of Hsp33 with oxidants such as HOCl causes massive conformational changes that are triggered by the formation of two intramolecular disulfide bonds and the release of the bound zinc ion (Figure 4.1.2).[84,85] Unexpectedly, structural studies revealed that activation of Hsp33 involves converting the protein from a stably folded, chaperone-inactive monomer into a partially unfolded chaperone-active dimer.[85–87] These features made Hsp33 the first known chaperone that gains activity through partial unfolding. Meanwhile, several other so-called conditionally disordered chaperones have been discovered that sense adverse environmental conditions, such as low pH or high temperature, through stress-induced unfolding and become activated in the process (for a review, see ref. 88).

Activation of Hsp33 is achieved by fast-acting oxidants such as HOCl or slow-acting oxidants such as peroxide when combined with protein unfolding conditions.[24,81,85] This specific set of activating conditions appears to restrict activation of Hsp33 to only those oxidative stress conditions that lead to protein unfolding. Consistent with these *in vitro* data, we found that deletion of Hsp33 causes a pronounced increase in bacterial HOCl or H_2O_2/heat sensitivity, concomitant with the accumulation of aggregated proteins *in vivo*.[24,81] Once activated, Hsp33 binds to a wide variety of different client proteins, engaging its intrinsically disordered regions directly in client interactions.[87,89] The complexes between Hsp33 and client proteins are apparently stable, and Hsp33 remains associated with unfolded clients until reducing

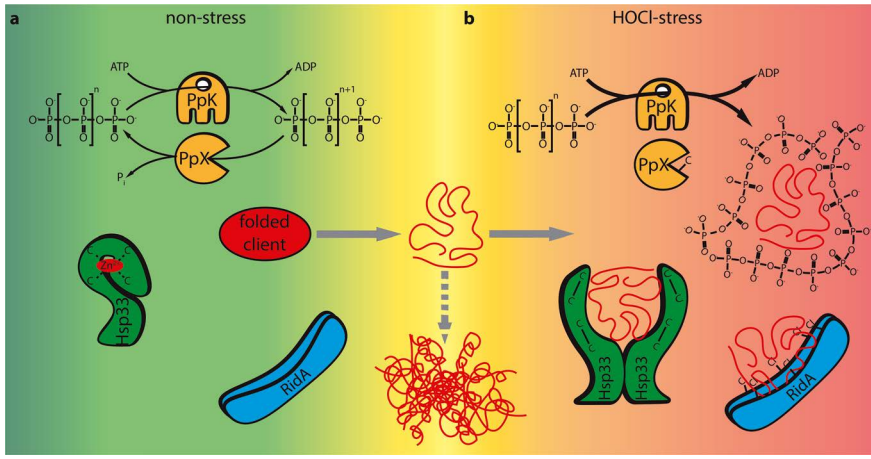

Figure 4.1.2 HOCl-dependent chaperone systems in *E. coli*. (a) Under non-stress conditions, the proteins Hsp33 and RidA are chaperone inactive and the cellular levels of polyphosphate (polyP) are kept low. Hsp33 is compactly folded using four absolutely conserved cysteines to coordinate one Zn^{2+} ion (dark green). RidA is a metabolically active enamine/imine deaminase (blue). PolyP levels are kept low through the regulation of PPK and PPX (orange). (b) HOCl treatment leads to global protein oxidation, unfolding and aggregation. At the same time, HOCl-dependent modifications activate the chaperone function of Hsp33 and RidA and convert large amounts of ATP into polyP. All three systems are highly effective in preventing toxic protein aggregation by binding aggregation-sensitive unfolding proteins. Activation of Hsp33 involves the formation of two intramolecular disulfide bonds, which causes massive conformational rearrangements, partial unfolding and dimerization (dark green). Activation of RidA is achieved by the *N*-chlorination of several positively charged residues, which increase RidA's surface hydrophobicity (blue). Accumulation of polyP is triggered in part by the oxidative inactivation of PPX (orange). All oxidative modifications are reversible and proteins and polyP levels return to their pre-stress conformation and concentrations once the stress is over.

conditions are restored and canonical chaperones are reactivated. Those then take over the client proteins from Hsp33 and refold them to their native state.[90] More details on the mechanism of Hsp33 activation, its regulation and the involvement of intrinsically disordered regions in client binding, complex stabilization and client release can be found elsewhere.[29,91]

4.1.4.3.2 RidA – Chaperone Activation by Methionine Oxidation

Originally described as enamine/imine deaminase,[92] the bacterial protein RidA is the newest member of a growing group of dual-function proteins, which switch from their regular enzymatic function under non-stress conditions to general ATP-independent chaperones under oxidative stress

conditions.[93] Unlike Hsp33, RidA's activation depends on the reversible *N*-chlorination of positively charged residues by HOCl and other RCS species (Figure 4.1.2). This chlorination of RidA appears to increase its surface hydrophobicity, which might promote its interaction with a range of aggregation-sensitive unfolding proteins.[93] Confirming its role in oxidative stress resistance, a deletion mutant of *ridA* was found to prolong the recovery phase following bleach treatment compared with wild-type cells.[93] *In vitro*, activation of RidA is rapidly reversed by thioredoxin, suggesting that the activation is a fully reversible process. It now remains to be investigated how chlorination of surface residues triggers chaperone activity and to what extent other proteins might use a similar mechanism to turn into molecular chaperones. The recent report that the unrelated eukaryotic α_2-macroglobulin also undergoes reversible *N*-chlorination to gain chaperone activity[94] suggests that this mechanism might be more widespread than previously anticipated.

4.1.5 Inorganic Polyphosphate: a Potent Protector Against Oxidative Damage

Polyphosphate (polyP) is a linear polymer consisting of a minimum of three and up to 1000 inorganic phosphate (P_i) molecules linked by high-energy ATP-like bonds. Discovered in yeast in the late 19th century, it was considered a "molecular fossil," a lost remnant in the evolution of polyanions.[95] The Nobel Laureate Arthur Kornberg revived interest in polyP in the mid-1950s, starting with the identification of the polyP-synthesizing enzyme polyphosphate kinase PPK in *E. coli*.[96] Since then, polyP has been identified in all studied organisms, where it adopts a variety of different subcellular structures, including acidocalcisomes in eukaryotic cells and volutin granules in bacteria.[97–99] The ancient nature of polyP together with its abundance in volcanic exudates and deep steam vents suggest a possible role in prebiotic evolution.[95]

Although structurally extremely simple, polyP has been reported to exert numerous seemingly unrelated biological functions, depending on its cellular localization, the cell type and the species. In bacteria and unicellular eukaryotes, polyP plays a role in biofilm formation, virulence, persistence, heavy metal tolerance, stress resistance and nutrient shift adaptation.[100–104] In mammals, polyP is involved in blood clotting, apoptosis, mTOR activation and neuronal signaling.[105–109] At this point, it is still unclear how polyP precisely affects these diverse processes. However, recent studies suggest that one common denominator might be polyP's ability to serve as a highly effective protein scaffold (see below for more details).

The polyP-synthesizing enzyme PPK and related homologs have been identified in various bacteria and some unicellular eukaryotes. However, for many more bacteria and also higher eukaryotic cells, the systems responsible for polyP production are still elusive.[110] PPK homologs, which have been extensively studied in *E. coli*, catalyze both the synthesis of polyP from ATP (PPK1) and the conversion of polyP back to ATP (*i.e.* PPK1,

PPK2).[96,99] Under non-stress conditions, polyP is mostly degraded into inorganic phosphate (P_i) through the processive activity of exopolyphosphatase (PPX) (Figure 4.1.2). Mutant strains lacking *ppk* show increased sensitivity towards numerous stress conditions, including ROS, RCS, heat and starvation.[111–115] In *E. coli*, *ppk* and *ppx* are present in the same operon under the control of heat-shock regulator σ^{32}.[116] Together, these observations have long pointed towards polyP being a central player in the cellular stress response. However, the mechanism by which polyP protects cells has remained largely elusive.

4.1.5.1 PolyP Functions as a Potent Chaperone

Previous studies revealed that polyP provides resistance towards a variety of different stress conditions (temperature, low pH, ROS, RCS), which have one feature in common, namely that they broadly damage proteins.[70,115] Based on these observations and the finding that mutant bacteria that lack polyP upregulate the heat-shock response when treated with protein unfolding oxidants such as HOCl[70] suggested that polyP might directly or indirectly affect protein solubility *in vivo*. Indeed, *in vitro* chaperone assays using purified proteins revealed that low micromolar concentrations of inorganic polyP prevented the irreversible aggregation of chemically or heat-denatured model substrates in a chain length-dependent manner.[70] *In vivo*, lack of polyP was found to increase dramatically the sensitivity of *E. coli* or *Pseudomonas aeruginosa* towards antimicrobial oxidants, including HOCl, HOBr and HOSCN, by failing to prevent the oxidant-induced aggregation of cytoplasmic proteins.[32,70] Like classic protein chaperones, polyP is able to maintain unfolding proteins in a refolding-competent conformation, and promote their refolding in the presence of chaperone foldases and ATP (Figure 4.1.2).[70] Unlike protein chaperones, however, polyP is redox inert, resistant towards ROS and RCS, and its production does not require time-consuming transcription or translation processes. These features allow polyP to provide protection rapidly under oxidative stress conditions.

4.1.5.2 PolyP Production During Oxidative Stress

Upon exposure to physiological oxidants, including HOCl and HOSCN, *E. coli* and *P. aeruginosa* rapidly accumulate large amounts of polyP at the expense of cellular ATP.[32,70] The fact that neither PPK nor PPX levels changed dramatically during stress suggested that polyP synthesis is post-translationally regulated. Indeed, studies in *E. coli* revealed that upon exposure to oxidative stress conditions, the polyP-hydrolyzing enzyme PPX is rapidly and reversibly inactivated through reversible oxidation of a key cysteine, which is located in the predicted polyP-binding site (Figure 4.1.2).[70] These results agreed with previous studies that showed that during the stringent response, which also causes a massive increase in polyP levels, PPX activity is transiently inhibited by the stringent response regulator guanosine 3′,5′-bisdiphosphate

(ppGpp).[117] It is of note, however, that cells lacking PPX contain equally low levels of polyP under non-stress conditions compared with wild-type cells, and are still able to trigger polyP accumulation in response to stress. These results suggest the presence of an additional PPK-related activation mechanism that contributes to stress-induced polyP production.[70]

4.1.5.3 The Many Other Hats of PolyP and Their Potential Roles in Oxidative Stress Protection

In addition to its direct role in oxidative stress resistance by serving as a molecular chaperone, polyP has several other reported functions in bacteria, including acting as modulator of the Lon protease, metal chelator and regulator of the general stress response protein RpoS (σ^{38}).[115,118] Since some (or all) of these processes might affect oxidative stress resistance in bacteria, we will review them briefly.

Almost 20 years ago, Kuroda and co-workers reported the finding that the accumulation of polyP upon nutrient shift triggers Lon protease-dependent degradation of proteins, particularly ribosomal subunits.[119] More recently, polyP was also found to stimulate the activity of the Lon-protease towards anti-toxin degradation.[101] The resulting imbalance between anti-toxin and toxin appears to push cells into dormancy, making them transiently more tolerant to antibiotic treatment, a process known as persistence (for a review, see ref. 120). At this point, it is unclear by what mechanism polyP affects Lon protease activity, and whether this function becomes effective during oxidative stress. It is conceivable, however, that activation of Lon protease might contribute to the degradation of oxidatively damaged proteins.

Another aspect of polyP function that might affect oxidative stress resistance is the finding that some metabolic enzymes, including glucokinase in *Mycobacterium tuberculosis* and NAD kinase in *Corynebacterium glutamicum*, can substitute ATP for polyP.[121–123] By using polyP, these enzymes continue to work even under ATP-depleted conditions of oxidative stress. Moreover, some proteins have been found to become polyP-phosphorylated on lysine residues, affecting their specific activities.[124] It remains be tested whether any of the functions of polyP play a beneficial or regulatory role during oxidative stress.

A third aspect that needs attention is the ability of polyP to chelate a variety of different metals, including manganese,[125] iron,[126] mercury and cadmium.[127] and also to facilitate copper export from the cell.[128] Some of these metals are directly released from their associated proteins during oxidative stress and, through Fenton-like reactions, can generate additional ROS.[129] It is, therefore, tempting to speculate that the chelating activity of polyP contributes to oxidative stress resistance.

Finally, polyP has been shown to affect transcriptional processes, which likely also contribute to oxidative stress protection. Previous studies revealed that polyP increases transcription of *rpoS*, the gene encoding σ^{38}, the main regulator of the general stress response system of *E. coli*.[130] The σ^{38} regulon

includes many ROS detoxifying genes, including *katE* (encoding the peroxide-detoxifying catalase), *sodC* (encoding superoxide dismutase) and *ppk* itself.[131] Given the many different roles by which polyP affects oxidative stress resistance in bacteria, it is safe to propose that polyP production is one of the major and defining events in bacterial oxidative stress protection.

4.1.6 Oxidative Stress-protective Systems as Novel Drug Targets

Production of high levels of ROS and RCS is a powerful strategy by which mammalian hosts defend themselves against invading pathogens. Not surprisingly, pathogens that lack the ability to withstand these oxidative insults are therefore much less able to cause infections. For instance, bacteria lacking polyP have been found to be strongly impaired in virulence, persistence and biofilm formation[101,104] and show increased sensitivity towards antibiotic treatment.[102] It is therefore not surprising that PPK, which does not have any homologs in higher eukaryotes, has long been proposed to serve as a potentially powerful novel antimicrobial drug target.[95] Limiting the success of finding PPK inhibitors, however, was the previously established PPK activity assay that was incompatible with high-throughput screens. Our laboratory recently established a more suitable assay, and succeeded in identifying 5-aminosalicylic acid (mesalamine) as a compound that actively inhibits bacterial PPK *in vitro* and substantially reduces polyP accumulation *in vivo*.[132] Mesalamine, an FDA-approved drug and the gold standard in treating ulcerative colitis, has long been known to reduce inflammation, and has recently been found to restore a healthy gut microbiome.[133] We have now discovered that mesalamine treatment of a wide variety of PPK-containing pathogens, including *Vibrio cholera*, uropathogenic *E. coli* (UPEC) and *P. aeruginosa* faithfully reproduced phenotypes previously associated with *ppk* deletions: (i) increased sensitivity towards antimicrobial oxidants including HOCl, HOBr and HOSCN; (ii) decreased persistence towards antibiotics; (iii) delayed biofilm formation; and (iv) reduced colonization ability.[32,132] These results suggest that mesalamine treatment might affect pathogen survival within a chronically inflamed environment by sensitizing the bacteria towards host-induced oxidants. Much needs to be done to test this idea, but the tools are available and the potential to develop alternative new antimicrobial drugs is certainly a powerful incentive.

4.1.7 Concluding Remarks

The bacteria's response to reactive oxygen species has been extensively studied over the past 30 years. In contrast, much less was known about the cellular effects of HOCl and related compounds, despite their important antimicrobial roles and high toxicity.[19,134] One possible reason why RCS have been neglected for so long is the fact that they are, indeed, very highly reactive and

not easy to work with.[30] Nevertheless, over the past few years, the focus has shifted and we now know of at least nine different HOCl-specific transcriptional regulators and three chaperone systems that are employed by bacteria to prevent RCS-mediated damage. One of the most powerful protective systems identified so far appears to be polyP, which takes on a central role as both regulator of and actor in the oxidative stress response. Future goals are to shed light on the molecular mechanisms by which polyP functions as a chaperone. Specifically, we need to understand how polyP recognizes protein-folding intermediates, stabilizes unfolding proteins and selects its substrates. From a more general point of view, it is essential to identify the polyP-synthesizing enzymes in Gram-positive pathogens, such as *Staphylococcus aureus*, and determine the polyP machinery in higher eukaryotes. Recent studies revealed that polyP acts as a signaling molecule controlling inflammation in mammals,[135] and that bacteria can modulate inflammatory responses *via* polyP secretion.[136] As long as we do not know how polyP is made and degraded, we remain in the dark about its role in mammalian oxidative stress protection. These future studies will provide us with potentially new ways to treat infection and potentially other human diseases. As Arthur Kornberg said years ago, "*It is our responsibility to show why polyphosphate is important.*"[137] He truly lived up to this responsibility. It is now up to us to continue his legacy.

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