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Oxidative Stress Protection by Polyphosphate - New Roles for an Old Player

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Abstract

Inorganic polyphosphate is a universally conserved biopolymer, whose association with oxidative stress resistance has been documented in many species, but whose mode of action has been poorly understood. Here we review the recent discovery that polyphosphate functions as a protein-protective chaperone, examine the mechanisms by which polyphosphate-metal ion interactions reduce oxidative stress, and summarize polyphosphate's roles in regulating general stress response pathways. Given the simple chemical structure and ancient pedigree of polyphosphate, these diverse mechanisms are likely to be broadly relevant in many organisms, from bacteria to mammalian cells.

INTRODUCTION

Oxidative stress is an inevitable consequence of aerobic life. Reduction of molecular oxygen generates a series of reactive oxygen species (ROS), including superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^\bullet$). ROS are capable of damaging proteins, DNA, lipids, and other cellular components, and exert powerful, and under some conditions, potentially lethal stress on bacterial cells [1,2]. The types of damage caused by each of these species and the mechanisms by which bacteria defend themselves have recently been reviewed [3]. Many bacterial defenses against oxidative stress are based on changes in gene expression, upregulating the cellular concentration of enzymes that scavenge superoxide and hydrogen peroxide or repair oxidative damage to DNA and iron-sulfur clusters in proteins. In addition, however, several post-translational response mechanisms have been identified, involving the redox-regulated adjustment of cellular metabolism to oxidative stress conditions, the activation of specific molecular chaperones and the accumulation of

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inorganic polyphosphate (polyP). Together, these systems provide powerful protection against oxidative stress conditions in bacteria.

Inorganic polyphosphate is a polymer of phosphoanhydride-linked phosphate residues, found as chains up to 1000 residues long in cells from all three domains of life [4]. Despite its universal nature, the roles of polyP in cellular metabolism are only beginning to be understood. PolyP has long been known to affect the ability of a variety of prokaryotic and eukaryotic cells to resist oxidative stress. Arthur Kornberg and coworkers showed in 1992 that disruption of the *Escherichia coli ppk* gene that encodes polyP kinase (PPK), the enzyme catalyzing the reversible synthesis of polyP from ATP [5], resulted in increased sensitivity towards multiple stressors, including heat, starvation, and H_2O_2 [6]. Since then, *ppk* mutants of many species of bacteria have been shown to be sensitive to ROS treatment (for examples, see [4,7–10]). However, until very recently, the molecular mechanisms by which polyP protects cells against oxidative stress have been uncertain. An increased understanding of the molecular mechanisms by which polyP acts is likely to have broad implications, especially considering recent developments showing that polyP is a signal molecule controlling inflammation in mammals [11] (a process that involves production of considerable ROS) and that bacterial polyP can modulate host inflammatory responses [12,13].

In this review, we discuss exciting advances of the past three years that have demonstrated mechanistic details of multiple pathways by which polyP both directly and indirectly protects bacteria from oxidative stress. These range from direct protein-stabilizing chaperone activity, to interactions with redox-active metals, to regulatory roles in controlling stress response pathways.

POLYPHOSPHATE IS A PROTEIN-PROTECTING CHAPERONE

Hypochlorous acid (HOCl), the active ingredient of household bleach, is an extremely potent microbicidal oxidant whose effects on bacterial cells we have recently reviewed [14]. HOCl is notable among ROS for causing extremely rapid protein damage by oxidizing cysteine, methionine, histidine, and other amino acids, leading to unfolding and aggregation of oxidized proteins [15]. A recent study of the *E. coli* response to HOCl showed that treatment of bacteria with sublethal doses of HOCl caused the rapid accumulation of polyP, and that *E. coli* mutants lacking PPK were exquisitely sensitive to HOCl treatment [8]. These observations led to the question of how polyP was protecting cells against HOCl, and to the unexpected discovery that polyP interacts directly with unfolding proteins and prevents their aggregation, acting as an inorganic chaperone (Figure 1). Physiological concentrations of polyP were able to efficiently prevent protein aggregation both *in vivo* and *in vitro*, with longer chains of polyP exerting a stronger protective effect than shorter chains. PolyP was able to protect a broad spectrum of proteins from aggregation, suggesting that this chaperone function is very likely to be important in any cell that accumulates polyP. Moreover, and in true protein chaperone-like fashion, polyP maintained proteins in a refolding competent conformation, handing its clients over for successful refolding by the DnaK/DnaJ/GrpE chaperone machinery. PolyP synthesis is directly regulated by HOCl via reversible oxidative inactivation of the polyP-degrading exopolyphosphatase PPX [8],

allowing it to bypass time-consuming transcription/translation processes. In addition, PolyP has several other distinct advantages over more conventional protein chaperones under severe oxidative stress conditions. Unlike proteins, polyP does not react with HOCl or other ROS [15]. It does not require ATP for its function and in fact, upon release of stress, polyP can be efficiently converted back into ATP by PPK [5], which can then be used by ATP-dependent chaperones to promote protein refolding [8].

Many questions about the chaperone function of polyP remain unanswered. How does polyP interact with unfolding proteins to prevent their aggregation? To what extent does loss of polyP chaperone function explain the pleitotropic phenotypes of *ppk* mutants in bacteria, which are commonly sensitive to multiple stresses and defective in motility, biofilm formation, and virulence [4]? What characterizes the client proteins of polyP, and is there an overlap between polyP substrates and the clients of other molecular chaperones?

MANGANESE-PHOSPHATE DETOXIFIES SUPEROXIDE

Aerobic organisms contain multiple superoxide dismutase enzymes which scavenge O_2^-, preventing inactivation of essential iron-sulfur cluster and mononuclear iron-containing enzymes [3]. However, some aerotolerant anaerobes, such as the lactic acid bacterium *Lactobacillus plantarum,* do not contain superoxide dismutase [16,17]. As reviewed recently [17], these organisms compensate for the loss of enzymatic superoxide dismutase by accumulating high concentrations of Mn^{2+} ions which, when coordinated by a variety of organic and inorganic metabolites, are able to detoxify O_2^-. In *L. plantarum*, non-enzymatic superoxide dismutase activity was originally (in 1982) associated with Mn^{2+}-polyP complexes [16], indicating that polyP might play a role in this process. The more recent finding that *Saccharomyces cerevisiae* mutants lacking superoxide dismutase can be rescued by addition of manganese, but mutants lacking both superoxide dismutase and the polyP polymerase Vtc4 cannot [18,19], further reinforced this association of polyP with Mn^{2+}-dependent antioxidant activity.

Until very recently, the physiologically relevant ligand(s) and chemical mechanism of Mn^{2+}-dependent superoxide scavenging were controversial. In 2012, however, Valentine and coworkers provided experimental proof that manganous phosphate ($MnHPO_4$) and manganous carbonate ($MnCO_3$) catalyze superoxide dismutation at rates competitive with enzymatic superoxide dismutase [20]. In contrast, they found that manganous pyrophosphate ($MnP_2O_7^{2-}$) reacted only stoichiometrically with O_2^-, resulting in non-catalytic detoxification of O_2^- and oxidation of Mn^{2+} to Mn^{3+}. A dual, catalytic and non-catalytic model would explain the mechanism(s) behind the observed role of polyP in managnese-dependent detoxification of O_2^- (Figure 2). Since polyP efficiently coordinates divalent cations like Mn^{2+} [21], it inevitably stabilizes the cellular Mn^{2+} pool. Hydrolysis of Mn^{2+}-polyP by PPX will then generate $MnHPO_4$, which will rapidly and catalytically detoxify O_2^- (Figure 2A). Alternatively, and in analogy to the reaction observed with $MnP_2O_7^{2-}$, Mn^{2+}-polyP may non-catalytically detoxify O_2^-, resulting in accumulation of Mn^{3+}-polyP (Figure 2B). In either case, the cell is efficiently protected from ROS without the need for superoxide dismutase. To test this model, however, future experiments are necessary that

examine the role of PPX in O_2^- detoxification and measure levels of Mn^{3+} in O_2^--stressed bacteria.

POLYPHOSPHATE DEFENDS AGAINST THE FENTON REACTION

Reaction of H_2O_2 or HOCl with redox-active metal ions like Fe^{2+} or Cu^{2+} generates the highly reactive and toxic hydroxyl radical ($OH^•$) via the Fenton reaction [3]. At least two distinct mechanisms explain how polyP might reduce the cytotoxic effects of the Fenton reaction. *In vitro* studies have been shown that PolyP dramatically reduces the $OH^•$ yield of the Fenton reaction (Figure 2C) [22], despite the fact that chelation of Fe^{2+} by polyP accelerates the rate of the Fenton reaction by several orders of magnitude [23]. The explanation for this apparent contradiction appears to lie in the ability of polyP to stabilize the Fe^{3+} intermediate, thereby inhibiting the regeneration of Fe^{2+}, which is necessary for additional cycles of $OH^•$ generation [23]. However, additional work is needed to validate this model *in vivo*. In addition, polyP has been shown to facilitate export of Cu^{2+} from the cell (Figure 2D). The details of this mechanism have recently been elucidated in *E. coli*, where Cu^{2+} tolerance was shown to depend on polyP synthesis by PPK, polyP degradation by PPX, and the metal-phosphate symporters PitA or PitB [24], supporting a model in which Cu^{2+} is first chelated by polyP to reduce its toxicity, followed by PPX cleavage of PO_4^{3-} from polyP and co-export of the resulting PO_4^{3-} and Cu^{2+} via the Pit system. This model is consistent with earlier work on Cu^{2+}-resistance in *Sulfolobus metallicus* [25] and both Cd^{2+} and Hg^{2+} resistance in *E. coli* [26,27]. These studies indicate that polyP's involvement in metal export is conserved very broadly among prokaryotes and that it is not limited to the Fenton reaction-catalyzing metal Cu^{2+} but applies also to other metals like Cd^{2+} or Hg^{2+}, which are known to oxidize cysteine residues [28].

POLYPHOSPHATE IS REQUIRED FOR FORMATION OF PERSISTER CELLS

Persister cells are members of a bacterial population that stochastically enter a dormant state, in which they become highly stress-resistant (reviewed recently in [29]). Although this phenomenon has been studied largely in the context of antibiotic resistance, formation of persister cells is also stimulated by ROS [30]. PolyP has recently been shown to play a central role in the molecular mechanism by which bacteria enter this stress-tolerant state (Figure 3A) [31]. Persister cells arise due to stochastic accumulation of high levels of the alarmone (p)ppGpp in a subpopulation of cells. Since(p)ppGpp has been shown to inactivate the polyP-degrading enzyme PPX [32], polyP accumulates. PolyP, in turn, is thought to interact with the Lon protease, stimulating digestion of a subset of cellular proteins [33]. These proteins include the antitoxin modules of Type II toxin-antitoxin systems [31]. The free toxins (*e.g.* HipA, RelE, MazF, *etc.*) then go on to inhibit cell metabolism at a variety of levels, including transcription, translation, and DNA replication, with the result that affected cells enter a slow-growing persister state [29]. Populations of mutants lacking PPK form dramatically lower levels of persister cells, reinforcing the importance of polyP in control of this stress response mechanism [31].

POLYPHOSPHATE IS INVOLVED IN REGULATION OF GENERAL STRESS RESPONSE NETWORKS

It has been known for some time that polyP plays a role in regulation of the σ^{38}-dependent general stress response system of *E. coli* (Figure 3B) [34–37]. This conclusion was based on the observation that polyP is required for efficient transcription of *rpoS*, the gene encoding σ^{38} [36], but the mechanism by which this occurs remains mysterious, and is an intriguing question for future research. The σ^{38} regulon includes many genes encoding enzymes important for ROS resistance, including *katE* (encoding catalase), *sodC* (encoding superoxide dismutase), and *ppk* itself [38]. A mutant lacking σ^{38} does not accumulate polyP in response to osmotic stress or nitrogen starvation [34], indicating the presence of a feedback loop between polyP and *rpoS* expression in *E. coli*. Evidence for a similar role of polyP in control of *rpoS* expression in *Pseudomonas putida* has recently been published [10].

More recently, studies in *Mycobacterium* spp. have also demonstrated a role for polyP in the regulation of sigma factor σ^{E}, which controls a stress-responsive regulon required for virulence, persistence in macrophages, and resistance to a variety of stresses, including oxidative stress and phosphate starvation [39,40]. Many of the components of this regulatory loop and the resulting stress-resistance phenotypes are conserved among even distantly related bacteria (Figure 3C). It has been shown that the correct control of this regulon depends on the ability of *M. tuberculosis* to synthesize polyP and the two-component transcriptional regulatory system MprAB, which activates *sigE* expression in response to polyP [41,42]. ROS-dependent activation of σ^{E} activity is mediated by the redox-sensitive anti-sigma factor RseA [43], and leads to the expression of *rel* (encoding the (p)ppGpp synthase RelA), *ppk1* (encoding the *M. tuberculosis* polyP kinase PPK1), and *mprAB.* These gene products help to increase the amount of polyP in the cell [40,41] and control the circuit.

While evidence from a number of bacteria supports the involvement of polyP in regulation of general stress responses, the precise details appear to differ somewhat among species. Common themes include regulatory loops in which polyP is required for expression of sigma factors that drive *ppk* gene expression, coordinated roles for polyP and the alarmone (p)ppGpp, and a central role for polyP in regulatory circuits controlling stress-resistance enzymes, including those that detoxify ROS (Figure 3). Determining the exact mechanism by which polyP regulates gene expression and the extent of conservation of these pathways in different bacteria represent interesting avenues for future research.

CONCLUSIONS

Exciting progress has been made in the last three years in the understanding of how polyP contributes to oxidative stress resistance, and has highlighted the profoundly multifunctional nature of this ancient biopolymer. Polyphosphate has multiple functions that increase bacterial oxidative stress resistance. As a chaperone, polyP prevents aggregation of damaged proteins. As a metal chelator, polyP facilitates detoxification of superoxide, reduces the free radical yield of the Fenton reaction, and promotes export of toxic redox-active metals. PolyP is also intimately involved in regulation of general stress response pathways in different

bacteria, including those that lead to stress-resistant persister cells and to expression of a variety of antioxidant enzymes. While this multifunctionality complicates the interpretation of experiments, requiring careful controls to distinguish which mechanism(s) are playing the most important role(s) under specific conditions, it goes a long way towards explaining the diverse and pleiotropic phenotypes associated with polyP.

Acknowledgments

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HIGHLIGHTS

- Polyphosphate protects bacteria from oxidative stress by multiple mechanisms.
- Polyphosphate is a chaperone that prevents aggregation of oxidized proteins.
- Polyphosphate-metal complexes lower levels of ROS and redox-active metals in cells.
- Polyphosphate is a key regulator of persister cell formation.
- Polyphosphate is involved in regulation of general stress response networks.

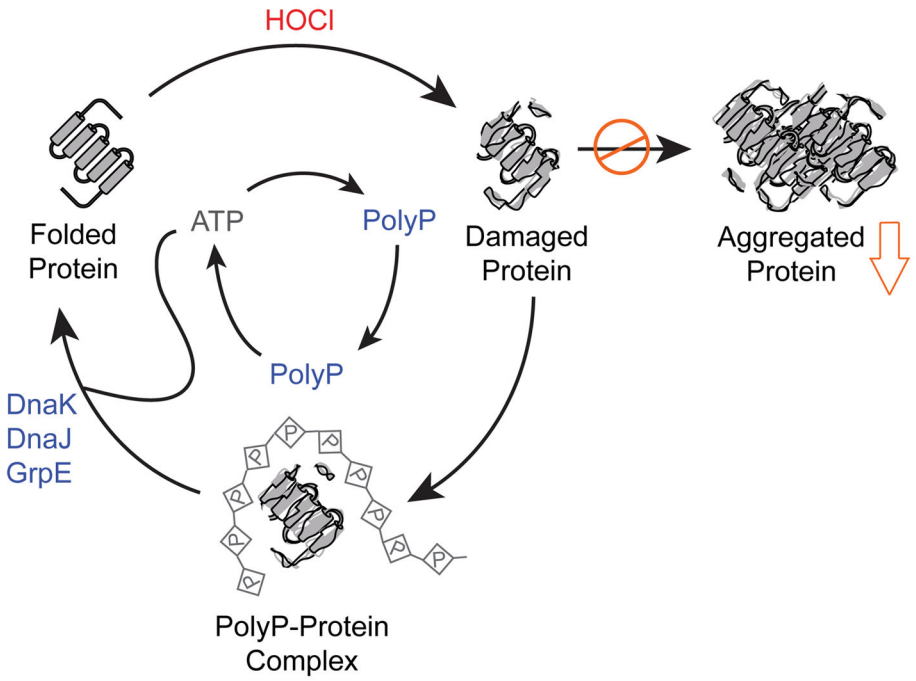

Figure 1.

Polyphosphate acts as a chaperone to prevent aggregation of oxidatively damaged proteins. Proteins damaged by oxidation, especially by the strong oxidant HOCl, are prone to cytotoxic aggregation. PolyP, generated from ATP under oxidative stress conditions, forms stable complexes with unfolding proteins, keeping them soluble and competent to be refolded. Upon relief of stress, polyP can be reconverted to ATP, which can then be used by ATP-dependent chaperones (*e.g.* DnaK, DnaJ, and GrpE) to refold polyP-protected proteins. Modified with permission from [8]. Abbreviations: HOCl, hypochlorous acid; ATP, adenosine triphosphate; polyP, inorganic polyphosphate.

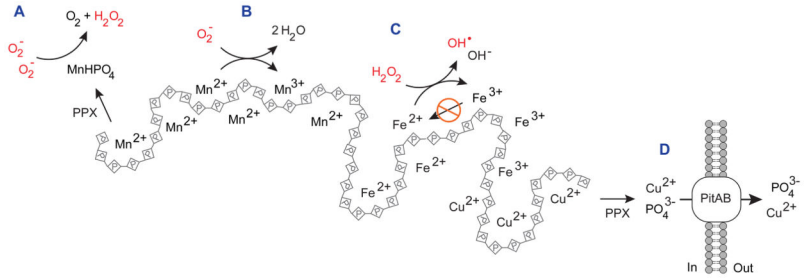

Figure 2.

Polyphosphate-metal complexes play multiple roles in oxidative stress resistance. **(A)** $MnHPO_4$, likely to be generated by PPX digestion of manganese-polyP complexes, catalyzes dismutation of superoxide to O_2 and H_2O_2. **(B)** Mn^{2+} ions in complex with polyP can non-catalytically quench superoxide, yielding Mn^{3+} and H_2O. **(C)** Complex formation between polyP and redox active metals (*e.g.* Fe^{2+}, Cu^{2+}) reduces the yield of hydroxyl radicals by slowing regeneration of the Fenton-reactive Fe^{2+}. **(D)** PolyP facilitates export of Cu^{2+} via a process requiring PPX and the metal-phosphate symporters PitA and PitB. Abbreviations: PPX, exopolyphosphatase; H_2O_2, hydrogen peroxide; $OH^{\bullet}$, hydroxyl radical; O_2^-, superoxide.

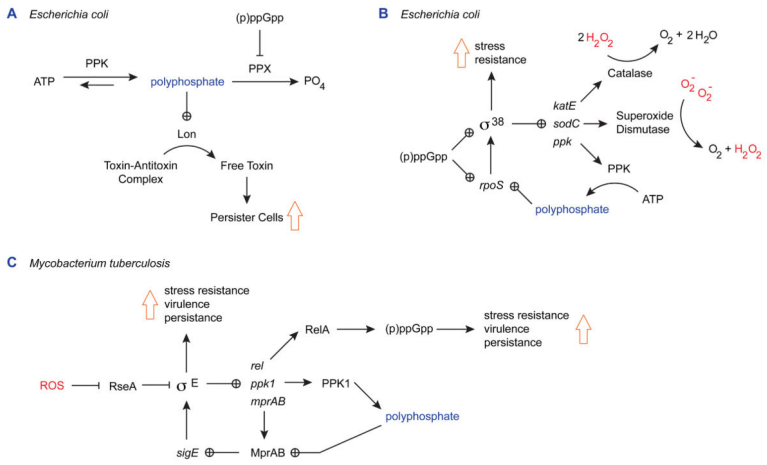

Figure 3.

Polyphosphate regulates general stress response networks. **(A)** In *E. coli*, the stress alarmone (p)ppGpp inhibits PPX, stimulating accumulation of polyP. PolyP activates Lon protease, which then degrades the antitoxin components of type II toxin-antitoxin systems. Accumulation of free toxin reduces growth rate and leads to an increase in formation of broadly stress-resistant persister cells. **(B)** In *E. coli*, the general stress response regulon controlled by the sigma factor σ^{38} includes genes encoding PPK, catalase, and superoxide dismutase. PolyP activates expression of the *rpoS* gene encoding σ^{38}. **(C)** In *M. tuberculosis*, polyP is required for MprAB-dependent expression of the stress response sigma factor σ^{E}, which controls polyP and (p)ppGpp biosynthesis and is required for stress resistance, virulence, and persistence in macrophages. Oxidative stress (*e.g.* H_2O_2) leads to activation of σ^{E} by inactivating the σ^{E} anti-sigma factor RseA. Abbreviations: ATP, adenosine triphosphate; (p)ppGpp, guanosine penta-or tetra-phosphate; PPK and PPK1, polyphosphate kinase; PPX, exopolyphosphatase; H_2O_2, hydrogen peroxide; O_2^-, superoxide.