Role of inorganic polyphosphate metabolism in copper tolerance in *Sulfolobus metallicus*

Francisco Remonsellez, Alvaro Orell, and Carlos A. Jerez*

Laboratory of Molecular Microbiology and Biotechnology, Department of Biology, Faculty of Sciences, University of Chile, Santiago, Chile

*E-mail: cjerez@uchile.cl

Abstract

Polyphosphates (polyP) are ubiquitous molecules present in most organisms including bacteria, archaea and eukaryotes. Although several physiological functions have been attributed to polyP in addition to being a reservoir of phosphate, nothing is known about the possible role of polyP in archaea. A model for the participation of polyP in the tolerance to heavy metals has been proposed in bacteria. The intracellular concentrations of these ions would stimulate polyP degradation and the hydrolyzed phosphate obtained by the action of PPX could be transported out of the cell along with the metal cations. To study if such a system exists in *Archaea*, the presence of polyP was determined by the electron energy loss spectroscopy (EELS) procedure and quantified by using specific enzymatic methods in *S. acidocaldarius*, *S. metallicus* and *S. solfataricus*. All three microorganisms synthesized polyP during growth, but only *S. metallicus* greatly accumulated polyP granules. The differences in the capacity to accumulate polyP between these archaeans may reflect adaptive responses to their natural environment. Thus, *S. metallicus* was able to grow and tolerate up to 200 mM copper sulfate, with a concomitant decrease in its polyP levels with increasing copper concentrations. On the other hand, *S. solfataricus* could not grow in or tolerate more than 1-5 mM copper sulfate, most likely due to its low levels of polyP. Shifting *S. metallicus* cells to copper sulfate concentrations up to 100 mM showed a rapid increase in their PPX activity which was concomitant in time with a decrease in their polyP levels and a stimulation of phosphate efflux. Furthermore, copper in the range of 10 μM greatly stimulated PPX activity in cell-free extracts from *S. metallicus*. The results strongly suggest that a metal tolerance mechanism mediated through polyP is also functional in members of the *Sulfolobus* group.

Keywords: *Sulfolobus*, Polyphosphate metabolism, Copper tolerance

1. Introduction

Polyphosphates (polyP) are linear polymers of hundreds of orthophosphate residues linked by phosphoanhydride bonds that have a variety of physiological functions, such as serving as a reservoir of phosphate, substitution for ATP in kinase reactions, serving as a chelator of metals, and adaptation to different stress conditions [1]. The main enzyme involved in the biosynthesis of polyP is the polyphosphate kinase (PPK) that catalyzes the reversible conversion of the terminal phosphate of ATP into polyP [2]. An exopolyphosphatase (PPX) on the other hand, is known to hydrolyze polyP liberating inorganic phosphate (Pi) [3]. Many heavy metal resistance systems involve either an active efflux or a detoxification of metal ions by different transformations [4]. For copper, these include intracellular complexation, reduced accumulation, extracellular complexation or sequestration in the periplasm [5,6]. Van Veen [7] has shown that the inorganic phosphate transport system (Pit) in *E. coli* and *Acinetobacter johnsonii* can reversibly transport metal-
phosphates. Keasling & Hupf [8] using genetically engineered strains of *E. coli* obtained results indicating that not only a large quantity of intracellular polyP is important for tolerance to heavy metals but also the ability to synthesize and degrade polyP. Based on these results and those mentioned above, Keasling [9] proposed a model in which the intracellular cation concentration in bacteria would regulate the activity of PPX, which would in turn degrade polyP and the Pi generated accompanied by cation transport would be removed out of the cell through the Pit system.

Related to archaeal copper resistance mechanisms, some metal efflux pumps have been identified in the genomes of archaea [10]. The P-type CPX-ATPases are responsible for the transport of heavy metal ions in all kinds of organisms. Very recently, one of the two CPX-ATPases of *S. solfataricus* has been isolated and characterized [11]. In *Ferroplasma acidarmanus* the Fer1 copper resistance (cop) loci [12] which includes genes encoding a putative transcriptional regulator (copY), a putative metal-binding chaperone (copZ) and a putative copper-transporting P-type ATPase (copB) has been described [13]. By transcription analyses the authors demonstrated that copZ and copB are co-transcribed, and that the transcript levels increase significantly in response to exposure to high levels of Cu$^{2+}$ ions, suggesting that the transport system is operating for copper efflux.

In archaea, polyP has been reported only in *Methanosarcinae* [14] and in *Sulfolobus acidocaldarius* [15]. However, nothing is known about the possible role of polyP in archaea. A partially purified PPK was reported in *S. acidocaldarius* [15]. However, we demonstrated later that this protein was rather a glycogen synthase [16]. Despite the fact that no bacterial-type PPKs have been found in the majority of the available archaeal genomic sequences, we recently reported the isolation of a PPX enzyme from *S. solfataricus* [17]. To find out if a polyP mediated metal tolerance system was present in *Archaea*, in the present work we have studied the effect of copper on polyP metabolism in *S. acidocaldarius*, *S. metallicus* and *S. solfataricus*.

2. Methods

2.1 Strains and growth conditions.

*S. metallicus* DSM 6482, *S. acidocaldarius* DSM 639 and *S. solfataricus* DSM 1617 were grown as described by Remonsellez et al. [18]. To study copper tolerance of *S. metallicus* and *S. solfataricus*, the microorganisms were grown in their respective media, except different CuSO$_4$ concentrations ranging between 5 to 200 mM were present initially. For shift experiments, *S. metallicus* cells were grown in the absence of copper to the early stationary phase and after removing the medium from the cells by centrifugation, they were then shifted to a new medium containing 10, 20 or 100 mM CuSO$_4$. As a control, a shift of cells to 100 mM (NH$_4$)$_2$SO$_4$ was used. Aliquots were taken at different times and polyphosphate levels and PPX activity were determined [18].

2.2 PolyP quantification

PolyP was quantified by using a two-step conversion of polyP into ATP by polyphosphate kinase and quantification of ATP by using luciferase to generate light [18-19].
2.3 Preparation of cell-free extracts from \textit{S. metallicus} and assay of PPX activity

Control cultures grown to $10^8$ cells/ml or cultures shifted to different CuSO$_4$ concentrations or to 100 mM (NH$_4$)$_2$SO$_4$ were harvested by centrifugation (7,700 x g for 15 min). Then the cells were resuspended in 50 mM Tris-acetate (pH 7.0)-10 % sucrose buffer (20 µl per mg of wet weight), were frozen and sonicated eight times for 30 s each time. The lysate was centrifuged (4,300 x g for 5 min) to eliminate cellular debris, and the supernatant was used to measure PPX activity. PPX activity was determined as described before [17, 18]. One unit of enzyme was defined as the amount releasing 1 pmol of phosphate from polyP per min.

2.4 In vivo labeling of \textit{S. metallicus} with $^{32}$Pi and Pi efflux measurements

Cells were grown and labeled with $^{32}$H$_3$PO$_4$ in Pi sufficient condition (2 mM). Cells were collected by centrifugation and resuspended at a higher cell density ($10^{10}$ cells/ml) in a medium with a reduced Pi condition (0.2 mM Pi). To label the cells, $^{32}$H$_3$PO$_4$ [100 µCi/ml (3.7 MBq/ml)] was added and the microorganisms were further incubated for 20 h after which the radioactively-labeled cells were harvested by centrifugation. The $^{32}$Pi-labeled cells were exhaustively washed (five times) by resuspension and centrifugation with fresh medium containing 2 mM Pi to eliminate the non-incorporated radioactive label and finally were resuspended in the same medium to a cell density of $10^8$ cells/ml, in the presence or absence of CuSO$_4$. To determine the amount of $^{32}$Pi released to the medium, samples were taken periodically and the supernatants obtained by their centrifugation at 12,000 x g for 10 min were measured by scintillation counting and analysed by TLC on polyethylenemine-cellulose by using $^{32}$Pi as a standard [18].

3. Results and Discussion

3.1 Accumulation of polyphosphate in members of the genus \textit{Sulfolobus}

We have previously described the presence of abundant polyP granules in the acidophilic \textit{A. ferrooxidans}, which may be related to metal tolerance in this biomining bacterium [20]. To detect the presence of these granules in members of the genus \textit{Sulfolobus}, we analyzed by EM unstained cells of the microorganisms as shown in Fig. 1. When \textit{S. solfataricus} was observed (Fig. 1a) or \textit{S. acidocaldarius} (not shown), very faint and small granules, if any, were present. In contrast, the presence of two or three large electron-dense granules per cell was seen in \textit{S. metallicus} (Fig. 1b). By using electron microscopic microanalyses we demonstrated that the electron-dense granules accumulated by \textit{S. metallicus} were mainly composed by phosphate and most likely corresponded to polyP. To determine whether the electron-dense granules composed by phosphate were indeed due to a higher accumulation of polyP, we quantified polyP in \textit{S. metallicus}, \textit{S. acidocaldarius} and \textit{S. solfataricus} during their growth phases. All the microorganisms analyzed synthesized increasing amounts of polyP as growth proceeded, leveling off when the stationary growth phase was reached [18].
Fig. 1. Presence of electron-dense granules in members of the genus Sulfolobus. Cells of S. solfataricus (a) or S. metallicus (b) grown to the early stationary phase were analyzed by electron microscopy.

Accumulations of polyP in S. metallicus were remarkable for being so high and sustained in time compared to those in E. coli, which does not form visible polyP granules under common growth conditions [1]. Table 1 shows that the levels of 180 nmol polyP/mg of protein reached by S. metallicus at the stationary phase of growth are roughly 10-fold greater than those seen in S. solfataricus (20 nmol polyP/mg of protein) and S. acidocaldarius (10 nmol polyP/mg of protein). The amounts of polyP accumulated by S. metallicus are even higher than those reported (150 nmol polyP/mg of protein) for another microorganism accumulating polyP granules: Vibrio cholerae during the exponential phase of growth with a rich medium [21] and are closer to the 400 nmol/mg of protein accumulated by A. ferrooxidans [20]. It is possible that the polyP accumulations observed in these crenarchaeons contribute not only to their energy supply but also to their enhanced survival and stress resistance in the presence of metals, as suggested for bacteria [9, 20].

3.2 Effect of CuSO4 in the growth of S. metallicus and S. solfataricus

S. metallicus cells utilized in these experiments were previously adapted to grow at different concentrations of metals. S. metallicus could stand up to 200 mM Cu$^{2+}$ (Table 1) [18]. On the other hand, S. solfataricus could stand up to only 5 mM copper sulfate, in agreement with previous reports in which S. solfataricus was able to grow only in the presence of 0.1 to 1 mM range, being completely inhibited by growth in the presence of 10 mM copper [22]. This behaviour was similar to the one seen for S. acidocaldarius, which also did not grow in the presence of 10 mM Cu$^{2+}$ [22]. These results clearly contrast with the very high tolerance of S. metallicus to concentrations of copper sulphate as high as 100 and 200 mM.

Table 1. PolyP levels and copper tolerance in members of the genus Sulfolobus.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>PolyP (nmol Pi/mg protein)</th>
<th>Copper tolerance (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. metallicus</td>
<td>180</td>
<td>100-200</td>
</tr>
<tr>
<td>S. solfataricus</td>
<td>20</td>
<td>0.1-1</td>
</tr>
<tr>
<td>S. acidocaldarius</td>
<td>10</td>
<td>0.1-1</td>
</tr>
</tbody>
</table>
3.3 Effect of copper ions on the levels of polyP in *S. metallicus*

PolyP levels showed a gradual decrease with increasing concentration of copper in the growth medium of *S. metallicus* until a clear drop (more than 90%) was seen when the Cu²⁺ concentration was raised to 200 mM [18]. This behaviour correlated exactly with the disappearance of the polyP granules observed when cells were subjected to those high copper levels (Fig. 2a, b, c). Ninety six % of the cells contained 2 to 3 large polyP granules per cell in the absence of copper (Fig. 2a), whereas at 200 mM copper sulphate the granules were very small, with only 46 % of the cells containing only one of these aggregates (Fig. 2c). These results strongly indicate a possible relationship between the polyP levels and the adaptation of *S. metallicus* to grow in the presence of Cu²⁺.

A possible mechanism to explain the observed decrease in polyP levels when cells were exposed to copper ions was an increase of the PPX activity. When the effect of copper and other metals on the PPX activity present in cell-free extracts of non-adapted *S. metallicus* was determined, copper greatly stimulated PPX activity at very low concentrations. The same maximal PPX activity was reached both with copper and manganese. However, the metal concentration requirement for this was 10 μM for copper and 500 μM for manganese [18]. These results suggest a stimulatory effect of copper and other metal ions on PPX activity and polyP hydrolysis.

![Fig. 2. Reduction in polyP contents during exposure of *S. metallicus* to copper ions. Cells were harvested from cultures grown in the absence of CuSO₄ (a) or in the presence of 100 (b) or 200 mM (c) CuSO₄ and were analyzed by EM without staining. Arrows indicate some of the polyP granules. Values of polyP contents for each type of cell were determined enzymatically and are indicated at the bottom of each picture.](image)

3.4 PPX activity and polyP levels of non-adapted cells exposed to different copper concentrations

To further investigate the effect of copper ions on PPX activity and polyP levels, non-adapted cells were shifted to the presence of different CuSO₄ concentrations. Cells were collected at different time intervals and the PPX activity and polyP levels were measured in the cell-free extracts. A rapid increase in the PPX activity was seen when cells were exposed to 100 mM copper (Fig. 3). The increase in PPX activity corresponded in time with the decrease in polyP levels, strongly suggesting that the decrease in polyP levels observed when cells were shifted to the presence of copper was due to an increase of this PPX activity.
3.5. Effect of copper ions on the efflux of Pi from S. metallicus cells

The decrease in polyP levels due to increased PPX activity in S. metallicus cells subjected to Cu²⁺ should generate free Pi. To evaluate if this Pi was transported out of the cells, S. metallicus was grown to early stationary phase and labeled in vivo with ³²Pi. After washing the cells, they were resuspended in sulfur medium with or without Cu²⁺ ions and the Pi efflux was determined. A continuous basal release of label from the control cells was found. However, an increase in the Pi efflux over this basal level was observed when cells were exposed to Cu²⁺ ions [18].

Metal-phosphate complexes formation and transport have been demonstrated in cells or isolated membrane vesicles from bacteria by determinations of changes in the membrane potentials [23]. This type of system is not currently available in S. metallicus, which is the less known species of the Sulfolobus group. Our attempts to label S. metallicus cells with ⁶⁴Cu to determine the formation of metal-phosphate complexes were unsuccessful most likely due to competing protons in the acidic environment of this acidophile [18]. Until the formation of Cu-phosphate complexes is demonstrated in S. metallicus, we cannot rule out that copper may be exerting an indirect effect that alters polyP levels.

It is possible that the proposed model for polyP mediated metal detoxification in S. metallicus also operates in S. acidocaldarius and S. solfataricus. However, their very low levels of polyP synthesized compared to those in S. metallicus make this system less relevant in the former microorganisms, since they are very sensitive to copper [24] as we have also seen here. The proposed working model for metal ion detoxification based on the hydrolysis of polyP involves the transport of metal-phosphate complexes out of the cell (Fig. 4).

![Fig. 4. Model for polyP degradation and heavy metal transport in Sulfolobus.](image-url)
It has been proposed that the inorganic phosphate transport (Pit) system could be a possible candidate for this purpose, because it can reversibly transport metal-phosphate complexes [7, 9]. A Pit-like phosphate transport system was searched for in the available genomes of S. solfataricus, S. tokodaii and S. acidocaldarius. A Pit-like transporter was not found in these archaea, but instead we found an open reading frame coding for a protein similar to the Pho84 Pi transporter from S. cerevisiae. Experimental evidence indicates that yeast Pho84 transports metal-phosphate complexes, as Pit does (Persson et al., 2003). Pho84, like Pit, belongs to the family of Pi:H+ symporters and is a member of the major facilitator superfamily [26]. The Pho84 transporter is functional only in acidic environmental conditions [25]. Currently there is no evidence for a Pho84-like transporter in S. metallicus. However, it is interesting that only proteins similar to Pho84, were present in all the available genomes of acidophilic archaeca: Sulfolobus tokodaii, S. solfataricus, S. acidocaldarius, Thermoplasma acidophilum, T. volcanicum and F. acidarmanus [18].

Although a genomic DNA sequence is not available yet for S. metallicus, our results suggest that this archaeon might have a copper homeostasis similar to that of other microorganisms, but no experimental evidence supporting this proposal is available in S. metallicus. Irrespective of the possible existence of such metal cation uptake and efflux mechanisms, it is plausible that a polyP-mediated metal tolerance mechanism such as the one recently described for the metal resistant polyP-accumulating acidophilic bacterium A. ferrooxidans [20] is also of great functional survival for an extremophilic biomining archaeon such as S. metallicus.

4. Conclusion

Our results strongly suggest that a metal tolerance mechanism mediated through polyP is also functional in members of the Sulfolobus group. This ability to accumulate and hydrolyze polyP may not only play an important role in the survival of these microorganisms in sulfidic mineral environments containing high toxic metals concentrations but also in their applications in biomining.

5. References