Role of Inorganic Polyphosphate in the Energy Metabolism of Ticks

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1. Introduction

Inorganic polyphosphates are long chains of a few to several hundred phosphate residues linked by phosphoanhydride bonds (Figure 1). Polyphosphates have been found in all cell types examined to date and have been demonstrated to play diverse roles depending on the cell type and circumstances (Kornberg et al., 1999; Kulaev & Kulakovskaya, 2000). The biological roles played by polyphosphates have been most extensively studied in prokaryotes and unicellular eukaryotes, where they have been shown to regulate many biochemical processes including the metabolism and transport of inorganic phosphate, cation sequestration and storage (Kornberg et al., 1999), and membrane channel formation (Reusch, 1989; Jones et al., 2003), and they have also been found to be involved in cell envelope formation and bacterial pathogenesis (Rashid et al., 2000; Kim et al., 2002), the regulation of gene and enzyme activities (McInerney et al., 2006), the activation of Lon proteases (Kuroda et al., 2001), and KcsA channel regulation (Negoda et al., 2009).

Fig. 1. Inorganic Polyphosphate

Conversely, polyphosphate functions have not been extensively investigated in higher eukaryotes; however, there is a good deal of interest in polyphosphates in mitochondria regarding two circumstances: polyphosphate as a macroenergetic compound with the same energy hydrolysis of the phosphoanhydride bond as an ATP and, according to the endosymbiotic theory, mitochondria originated from ancient prokaryotic cells (Clements et

al., 2009; Kulakovskaya et al., 2010), thus, it would be intriguing to discover whether or not mitochondria have preserved polyphosphate functions such as the regulation of energy metabolism and the participation in transport channel formation.

2. Polyphosphate mobilization during *Rhipicephalus (Boophilus) microplus* embryogenesis

The tick *Rhipicephalus microplus* is a one-host tick that causes major losses to bovine herds, especially in tropical regions. In this scenario, major efforts have been made to develop immunoprophylactic tick control tools (Guerrero et al., 2006). Ticks are also vectors of parasites that cause hemoparasitic diseases, which are endemic in many cattle production areas (Sonenshine et al., 2006). *Rhipicephalus microplus* only has one host throughout all three life stages, usually a bovine animal, and a long feeding period (approximately 21 days). The adult female, after becoming completely engorged, drops off of the host and initiates oviposition approximately three days later. Being an oviparous creature, embryogenesis occurs in the absence of exogenous nutrients, and maternal nutrients are packaged in oocytes and mostly stored as yolk granules. Hatching occurs around 21 days after oviposition, and the emerging larvae can survive for several weeks before finding a host, using the remaining yolk as the only source of energy (Fagotto, 1990).

Early *R. microplus* embryonic stages are similar to those of *D. melanogaster* and mosquitoes (Bate & Arias, 1991; Monnerat et al., 2002). Tick embryogenesis is characterized by the formation of a non-cellular syncytium up to day 4 (Campos et al., 2006). After this, the embryo becomes a multicellular organism and starts organogenesis (Campos et al., 2006).

The function of polyphosphate as a phosphate reserve is well known in prokaryotes and also in eukaryote microorganisms (Kulaev & Vagabov, 1983; Kornberg, 1995; Kulaev, 2004). The cells of higher eukaryotes also carry polyphosphate, but in smaller amounts than found in microorganisms. Therefore, as well as being a source of phosphate, these biopolymers probably participate in regulatory processes (Kornberg et al., 1999). Total polyphosphate levels were quantified throughout *R. microplus* embryogenesis and the levels were found to be higher during embryo cellularization and segmentation, from the fifth to the seventh day of development, and declined after that until a plateau was reached. The free phosphate content rapidly decreased during syncytial blastoderm formation on the third day of development, and remained low until the twelfth day of embryogenesis, when it rapidly increased thereafter (Figure 2A). Exopolyphosphatase splits phosphate off from the end of a polyphosphate chain and represents one of the main enzyme types responsible for polyphosphate hydrolysis (Kulaev et al., 2004). The activity of exopolyphosphatase was analyzed during embryogenesis and its activity was in agreement with total polyphosphate mobilization (Figure 2B).

It is interesting to note that in *R. microplus* the decline in total polyphosphate content after the seventh day of embryogenesis did not reflect the increase in the free phosphate content, since this only occurs after the twelfth day, suggesting that polyphosphate also plays roles other than being a phosphate reserve for embryo development. In this case, an alternative source of phosphate could be derived from the dephosphorylation of vitellin, a major yolk protein that is gradually dephosphorylated throughout embryogenesis (Silveira et al., 2006). This source could mainly be used until segmentation of the embryo, on the seventh day of development, because there is no total polyphosphate mobilization during this period.

Fig. 2. Characterization of the total polyphosphate content during *R. microplus* embryogenesis. A) Total polyphosphate ($\blacktriangle$) was extracted and quantified and free phosphate ($\blacksquare$) was quantified in an egg homogenate on different days after oviposition. B) Total polyphosphate ($\blacktriangle$) was extracted and quantified and exopolyphosphatase activity ($\bullet$) was analyzed in an egg homogenate on different days after oviposition. Activity is expressed as units per milligram of total protein. The results represent the mean $\pm$ SD of three independent experiments, in triplicate.

Quantification of the major energy sources in the egg over the course of *R. microplus* embryogenesis suggests that lipids and carbohydrates are the main energy source used during early development of the embryo. The total lipid contents remained stable until the fifth day, dropped on the seventh day, and remained roughly unchanged until hatching (Figure 3A). The total sugar contents exhibited a similar pattern, although slightly delayed: the values remained stable until the seventh day, dropped on the ninth day and remained

constant until hatching (Figure 3B). This pattern suggests the utilization of lipids during the course of cellularization, a maternally driven process (Bate & Arias, 1993). On the other hand, carbohydrates would be the major energy source for the quick segmentation of the embryo, of zygotic nature (Nusslein-Volhard & Roth, 1989; Bate & Arias, 1993).

Fig. 3. Consumption of energetic sources. The major egg storage components of *R. microplus* were quantified on different days after oviposition. A) Lipid quantification, determined via the gravimetric method (Bligh & Dyer, 1959); B) total sugar concentration, measured using the method of Dubois (Dubois et al., 1956). The results represent the mean $\pm$ SD of three independent experiments, in triplicate.

Interestingly, mitochondrial polyphosphate utilization occurred during blastoderm formation and segmentation of the embryo, between the fifth and seventh days of development, and higher total polyphosphate utilization occurred after blastoderm formation and segmentation of the embryo, after the seventh day of development (Figure 4). Thus, mitochondrial polyphosphate levels seemed to correlate with the energy demand of the embryo during these developmental stages, during which the embryo utilized a large part of its reserve lipids and sugars.

Fig. 4. Polyphosphate metabolism during *R. microplus* embryogenesis. Polyphosphate levels during embryogenesis in the mitochondrial fraction (●) and total polyphosphate (■) during embryogenesis. The results represent the mean $\pm$ SD of three independent experiments, in triplicate.

2.1 Inorganic polyphosphate metabolism in tick mitochondria

Mitochondria from tick embryos in the segmentation stage (ninth day after oviposition) were isolated and respiration was measured using pyruvate as the substrate. The rate of oxygen consumption was 30 nmol/min/mg protein, and the respiratory control ratio (RCR) was 6.5. The process was KCN- and oligomycin-sensitive, this fraction exhibited an ATP hydrolyses azide sensitivity, a mitochondrial marker higher than 80%, and no activity of glucose-6-phosphate dehydrogenase, a cytosol marker, was detected (Table 1).

	State 3	State 4	RCR	% F - ATPase azide sensitive	G6PDH (U/mg protein)
Homogenate	---	---	---	49.50 %	2.9 $\pm$ 0.4
Mitochondrial fraction	30.2 $\pm$ 3.2	4.6 $\pm$ 0.7	6.5 $\pm$ 0.4	83.45 %	---

Table 1. Mitochondrial characterization

Once the mitochondria were characterized, mitochondria in eggs in the segmentation stage (ninth day after oviposition) were isolated and exopolyphosphatase activity was measured in order to evaluate the regulation of its activity. The influence of NADH, phosphate, and ADP was investigated in concentrations ranging from 0.1 to 2.0 mM. The activity of exopolyphosphatase was stimulated by a factor of two by NADH, whereas its activity was completely inhibited by 2 mM phosphate and slightly stimulated by ADP (Figure 5A). The activity of exopolyphosphatase was also measured during mitochondrial respiration using pyruvate as the substrate and polyphosphate as the only phosphate source. During this assay, the addition of a small amounts of ADP (0.2 mM) induced state 3 (phosphorylating respiratory rate) followed by state 4 (non-phosphorylating respiratory rate), when all of the ADP was converted to ATP.

Fig. 5. In (A), Mitochondrial exopolyphosphatase activity in *R. microplus* embryos. Mitochondria from eggs on the ninth day of embryogenesis were isolated and exopolyphosphatase activity was determined using polyphosphate₃ in the presence of 0.1–2 mM NADH, ADP and Pi. The results represent the mean $\pm$ SD of three independent experiments, in triplicate. B) Exopolyphosphatase activity was measured in the mitochondria of eggs on the ninth day of development during mitochondrial respiration with pyruvate as the oxidative substrate, polyphosphate₃ as the exopolyphosphatase substrate and oligomycin as ATP synthase. The activity is expressed as units per milligram of total protein and the results represent the mean $\pm$ SD of three independent experiments, in triplicate. The asterisk (*) denotes the difference between the populations and the significance was determined by a two-way ANOVA test (Kruskal-Wallis).

Thus, during state 3, a balance existed between the release of phosphate by exopolyphosphatase and ATP synthesis, since exopolyphosphatase activity was measured by the amount of phosphate present. The exopolyphosphatase activity increased during mitochondrial respiration when pyruvate and ADP were added. This increase did not occur without the addition of ADP, indicating that exopolyphosphatase is stimulated during state 3 and that the rate of phosphate release is higher than the rate of ATP synthesis. Indeed, the stimulatory effect was antagonized by oligomycin, an ATP synthase inhibitor (Figure 5B). These data suggest that mitochondrial exopolyphosphatase activity is regulated by phosphate and the energy demand.

Furthermore, it was possible to measure ADP-dependent mitochondrial oxygen consumption in the presence of polyphosphate and in the absence of any other phosphate source. This oxygen consumption was observed using polyphosphate₃ and polyphosphate₁₅; however, the consumption was higher with polyphosphate₃. On the other hand, heparin, an exopolyphosphatase inhibitor, blocked oxygen consumption, which was recovered when 5 mM phosphate was added and was again interrupted by the addition of oligomycin, an ATP-synthase inhibitor (Figure 6). These results suggest that polyphosphate was used as a phosphate donor for ATP synthesis due to the mitochondrial coupling observed when mitochondrial respiration was interrupted by oligomycin and the existence of membrane exopolyphosphatase in this process, due to the inhibition by heparin, which cannot cross the mitochondrial membrane and has its active site oriented toward the external face of the membrane. In fact, after mitochondrial subfractionation, the main exopolyphosphatase activity was recovered in the membrane fraction, supporting this hypothesis (Table 2).

Fig. 6. Polyphosphate as a source for ATP synthesis. Oxygen consumption was monitored using a reaction buffer in the absence of a phosphate source in the eggs on the ninth day of development. The addition of 1 mM ADP, 5 mM pyruvate, 0.5 μ M polyphosphate₃ and ₁₅, 20 μ g/mL heparin, 5 mM phosphate and 0.5 μ M oligomycin is represented in the figure. This experiment was repeated at least three times with different preparations, and this figure shows a representative experiment.

	Exopolyphosphatase activity (U / mg protein)	Heparin (% inhibition)
Mitochondria	0.60 ± 0.19	98
Soluble fraction (intermembrane space and matrix)	0.35 ± 0.06	98
Membrane fraction (mixture of inner and outer membranes)	1.11 ± 0.16	98

Table 2. Exopolyphosphatase activity in mitochondrial preparations. Exopolyphosphatase activity was measured using eggs on the ninth day of development using polyphosphate₃ as the substrate. The activity is expressed as units per milligram of total protein and the results represent the mean ± SD of three independent experiments, in triplicate.

2.1.1 A mitochondrial membrane exopolyphosphatase

Exopolyphosphatases have been found in prokaryotes and eukaryotes and, although in bacteria these enzymes mostly hydrolyze high molecular weight polyphosphates (Kumble & Kornberg, 1996), at least some of the enzymes from *Saccharomyces cerevisiae* and *Leishmania major* are more active in hydrolyzing short chain polyphosphates, such as polyphosphate₃ (Kumble & Kornberg, 1996; Rodrigues et al., 2002). Exopolyphosphatase from *Escherichia coli* requires divalent cations and K⁺ for maximum activity, while exopolyphosphatase from yeast only requires divalent cations (Lichko et al., 2003). Membrane mitochondrial exopolyphosphatase activity from the hard tick *R. microplus* was found to be stimulated by Mg²⁺ and was insensitive to K⁺. Only a few compounds that inhibit exopolyphosphatase have been identified (Kornberg et al., 1999): treatment with molybdate (a common phosphohydrolase inhibitor) and fluoride (a pyrophosphatase inhibitor) showed that exopolyphosphatase present in the mitochondrial membrane fractions was insensitive to these compounds. However, heparin, a good inhibitor of other well-characterized exopolyphosphatases (Lichko et al., 2003), was effective in almost 100% (Figure 7). In order to obtain an insight into membrane exopolyphosphatase kinetics, the apparent *K_m* was measured using polyphosphate₃ and polyphosphate₁₅ as substrates and the results were expressed as the average of three independent experiments. The membrane exopolyphosphatase affinity for polyphosphate₃ was 10 times stronger than for polyphosphate₁₅ (Table 3). These results are in contrast with those found in a mitochondrial membrane-bound exopolyphosphatase of *Saccharomyces cerevisiae*, in which case the affinity was stronger for long-chain polyphosphates (Lichko et al., 1998). However, the data demonstrated that membrane exopolyphosphatase kinetics were in agreement with the oxygen consumption rate, which was much higher for polyphosphate₃ than polyphosphate₁₅. These results reinforce the theory of coupling between the activity of this enzyme and mitochondrial ADP phosphorylation (Figure 8).

Substrates	<i>K_m</i> (μM)	<i>V_{max}</i> (μmol min ⁻¹ mg protein ⁻¹)
PolyP ₃	0.2	2.4
PolyP ₁₅	2.2	1.1

Table 3. Kinetics characterization of exopolyphosphatase activity in membrane preparations of mitochondria from *R. microplus* embryos on the ninth day of embryogenesis.

Fig. 7. The effect of some reagents on membrane exopolyphosphatase activity. Mitochondrial membrane fractions of *R. microplus* embryos in eggs on the ninth day of embryogenesis were isolated and the membrane exopolyphosphatase activity was determined using polyphosphate₃ as the substrate in the presence of 2.5 mM Mg²⁺, 50–200 mM K⁺, 10–100 μM molybdate, 1–10 mM NaF and 20 μg/mL heparin.

Fig. 8. Involvement of membrane exopolyphosphatase in mitochondrial respiration. Oxygen consumption was monitored using a reaction buffer in the absence of a phosphate source in the eggs on the ninth day of development in the presence of 1 mM ADP, 5 mM pyruvate, and 0.5 μM polyphosphate₃ and ₁₅. The results represent the mean ± SD of three independent experiments, in triplicate.

To further investigate the regulation of membrane exopolyphosphatase during mitochondrial respiration, the activity was measured using pyruvate as the substrate and polyphosphate as the only source of phosphate. Membrane exopolyphosphatase activity increased during mitochondrial respiration when pyruvate and ADP were added and the stimulatory effect was antagonized by potassium cyanide addition (decreased electron flux) and increased by protonophore carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (increased electron flux), suggesting that membrane exopolyphosphatase could be modulated by the electron flux (Figure 9). These findings are consistent with those of Pavlov et al., 2010, who demonstrated that the production and consumption of mitochondrial polyphosphate depends on the activity of the oxidative phosphorylation machinery in mammalian cells. Furthermore, heparin completely inhibited exopolyphosphatase activity, reinforcing the role of membrane exopolyphosphatase during mitochondrial respiration, and the respiration activation by membrane exopolyphosphatase activity indicated that exopolyphosphatase could be close to the site of ATP production.

Fig. 9. Regulation of mitochondrial exopolyphosphatase activity during mitochondrial respiration. The activity of exopolyphosphatase was measured in the mitochondria of the eggs on the ninth day of development during mitochondrial respiration, using pyruvate as the oxidative substrate, polyphosphate₃ as the exopolyphosphatase substrate, KCN as the respiratory chain inhibitor, FCCP as the un-coupler and heparin as the exopolyphosphatase inhibitor. The activity was expressed as units per milligram of total protein and the results represent the mean $\pm$ SD of three independent experiments, in triplicate. The asterisk (*) denotes the difference between the populations and the significance was determined by a two-way ANOVA test (Kruskal-Wallis).

Despite the regulation of membrane exopolyphosphatase by an increased or decreased electron flux, the sensitivity of this enzyme according to the redox state using polyphosphate₃ as the substrate was evaluated. The influence of 1.0 mM dithiothreitol (DTT) and 1.0 mM hydrogen peroxide (H₂O₂) was investigated at different times and the exopolyphosphatase activity was stimulated and inhibited by 50% of both, suggesting that exopolyphosphatase is tightly regulated by the redox state (Figure 10).

Fig. 10. The redox regulation of mitochondrial membrane exopolyphosphatase. Exopolyphosphatase activity was measured in the mitochondria of the eggs on the ninth day of development using polyphosphate₃ as the substrate. The mitochondria were treated with 1 mM DTT and 1 mM H₂O₂ for 0–20 min. The results represent the mean $\pm$ SD of three independent experiments, in triplicate.

Fig. 11. Polyphosphate quantification in the nuclear and mitochondrial fractions. Polyphosphate levels during embryogenesis in the nuclear fraction (■) and mitochondrial fraction (●) during embryogenesis. The results represent the mean $\pm$ SD of three independent experiments, in triplicate.

Additionally, mitochondrial polyphosphate can form polyphosphate/ Ca^{2+} /PHB complexes (Reusch, 1989) with ion-conducting properties similar to those of the native mitochondrial permeability transition pore (Pavlov et al., 2005). Polyphosphatases localized in the membrane can not only degrade, but they can also synthesize polyphosphate inside these complexes (Lichko et al., 1998). During the embryogenesis of *R. microplus*, the synthesis of polyphosphate occurs in mitochondria but not in the nuclei (Figure 11). As polyphosphate kinases have only been found in prokaryotes, the observation that polyphosphate synthesis in ticks only occurs in the mitochondrial fraction supports the possibility that such synthesis probably occurs via the action of these complexes, as already suggested for other organisms (Reusch and Sadoff, 1988; Lichko et al., 1998; Reusch et al., 1998; Abramov et al., 2007).

3. Conclusion

The ubiquity of polyphosphate and the variation in its chain length, location and metabolism indicate the relevant functions of this polymer, including those in animal systems. The present study showed that electron flux and the redox state may exert some influence on and be influenced by the activity of membrane exopolyphosphatase, and it describes a role for polyphosphate in the energy supply and ATP synthesis during embryogenesis of the hard tick *R. microplus*. In this sense, a more comprehensive understanding of polyphosphate biochemistry during tick embryo development may unravel additional targets that could be effective in the control of this ectoparasite and shed new light on polyphosphate metabolism.

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