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REVIEW

Inorganic Polyphosphates in Mitochondria*

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Abstract—Current data concerning the crucial role of inorganic polyphosphates (polyP) in mitochondrial functions and dysfunctions in yeast and animal cells are reviewed. Biopolymers with short chain length (~15 phosphate residues) were found in the mitochondria of *Saccharomyces cerevisiae*. They comprised 7-10% of the total polyP content of the cell. The polyP are located in the membranes and intermembrane space of mitochondria. The mitochondrial membranes possess polyP/Ca^{2+}/polyhydroxybutyrate complexes. PolyP accumulation is typical of promitochondria but not of functionally active mitochondria. Yeast mitochondria possess two exopolyphosphatases splitting P_i from the end of the polyP chain. One of them, encoded by the *PPX1* gene, is located in the matrix; the other one, encoded by the *PPN1* gene, is membrane-bound. Formation of well-developed mitochondria in the cells of *S. cerevisiae* after glucose depletion is accompanied by decrease in the polyP level and the chain length. In *PPN1* mutants, the polyP chain length increased under glucose consumption, and the formation of well-developed mitochondria was blocked. These mutants were defective in respiration functions and consumption of oxidizable carbon sources such as lactate and ethanol. Since polyP is a compound with high-energy bonds, its metabolism vitally depends on the cell bioenergetics. The maximal level of short-chain acid-soluble polyP was observed in *S. cerevisiae* under consumption of glucose, while the long-chain polyP prevailed under ethanol consumption. In insects, polyP in the mitochondria change drastically during ontogenetic development, indicating involvement of the polymers in the regulation of mitochondrial metabolism during ontogenesis. In human cell lines, specific reduction of mitochondrial polyP under expression of yeast exopolyphosphatase PPX1 significantly modulates mitochondrial bioenergetics and transport.

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Key words: inorganic polyphosphate, mitochondria, polyP/Ca^{2+}/polyhydroxybutyrate, exopolyphosphatase, membrane potential, glucose repression, yeast, animal, insect

The inorganic polyphosphates (polyP), linear polymers of orthophosphoric acid, perform in living cells multiple functions including phosphate and energy reservation, formation of membrane channels, gene activity control, and participation in stress overcoming [1-8]. Interest in these compounds present in mitochondria is associated with two circumstances. In the first place, polyP is a macroergic compound so that the energy of phosphoanhydride bond hydrolysis is the same as in ATP. Consequently, in mitochondria with energy transformation as the primary function, they could participate in its regulation. In the second place, according to the endosymbiotic theory [9-14], which is currently supported by more and more facts [13, 14], mitochondria originated from ancient prokaryotic cells. Hence, the intriguing question is whether the mitochondria have preserved the functions of polyP such as regulation of energy metabolism, participation in transport channel formation, and in overcoming of stress conditions.

Finally, quite a number of works show that the synthesis of pyrophosphate, the simplest inorganic macroergic phosphorous compound, takes place in mitochondria [15-18]. Pyrophosphate is metabolized by a special set of enzymes, to a certain extent independently of the pathways of polyP metabolism [19, 20]. However, pyrophosphate is employed in the pathways of polyP metabolism, because it is formed during their utilization [21]. Evidently it acts as a primer during their synthesis [22]. One should mention the reviews [15, 16] considering the problem of pyrophosphate biosynthesis in mitochondria, especially under unfavorable impacts on these organelles,

Abbreviations: PHB, polyhydroxybutyrate; polyP, inorganic polyphosphates.

* On March 26, 2010 Igor Stepanovich Kulaev celebrated his 80th anniversary. The coauthors congratulate him with warm wishes of health and success in science.

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and the works [12, 19] on the possible role of H^+-PPases in the transformation of membrane potential energy into chemical energy of the phosphoanhydride bond and evolution of proteins functioning as proton pumps.

In the present review we will consider the data on relatively long-chain polyPs in mitochondria and present results demonstrating their crucial role in mitochondrial functions and dysfunctions in yeast and animal cells.

PolyP IN MITOCHONDRIA: DETECTION, QUANTITY, CHAIN LENGTH

For a long time it was considered that mitochondria contained pyrophosphate but lacked higher molecular weight inorganic polyP, because they could not be detected by traditional methods of chemical extraction and analysis [1].

Some of the early data on the probable participation of polyP in Ca-buffering, labile phosphate metabolism, and mineralization in mitochondria in animal tissues, especially in bones and teeth, are summarized in review [7]. Two types of electron-dense bodies were identified by electron microscopy in the mitochondria of rat liver, heart, and muscle: crystals similar to those in bones and teeth and amorphous aggregates. The reported characteristics suggest polyP to be components of amorphous, electron-dense, calcium- and phosphate-containing granules, which are unstable in acids [7].

Later on, more perfect methods of polyP analysis were developed. They made it possible to ascertain the presence of these polymers in mitochondria, though usually in small amounts.

The polyP in rat liver mitochondria [23] was estimated by an effective enzymatic method with purified polyphosphate kinase from *E. coli*. Its content was similar to that in the cytosol.

In the mitochondria of *Saccharomyces cerevisiae*, polyP was first observed during ^{31}P-NMR study [24]. The polyP extracted from mitochondria preparations by perchloric acid had an average chain length of 14 residues and corresponded to 10% of the total NMR-detectable polyP of yeast cells [24].

Table 1. Levels of polyP extracted by 0.5 N $HClO_4$ in mitochondria of *S. cerevisiae* (wild strain VKM Y-1173) under different growth conditions [25-27]

Growth conditions	PolyP, μmol P/mg protein
P_i limited medium (1 mM P_i), glucose	0.022
Complete medium (20 mM P_i), glucose	0.24
Phosphate overplus, glucose	0.44
Phosphate overplus, ethanol	0.10

Later, the polyP from isolated mitochondria of *S. cerevisiae* was studied by chemical extraction with 0.5 N $HClO_4$ and electrophoresis in polyacrylamide gel [25-27]. Promitochondria with active ATPase but decreased respiration level were obtained from cells grown under glucose repression [25, 26]. They contained polyP at a level strongly dependent on P_i concentration in the culture medium (Table 1). The level of acid-soluble polyP was the most variable. Salt-soluble and acid-insoluble polyP levels were no more than 10% as compared with the level of acid-soluble polyP.

A usual experimental model for investigation of polyP metabolism in yeast includes the conditions of phosphate limitation and so-called phosphate overplus, when P_i starvation and further transfer of the cells to a complete medium induce significant polyP accumulation [28]. Such a model was used for study of polyP in mitochondrial fraction isolated from *S. cerevisiae* cells grown on glucose [25]. Under P_i limitation, the level of mitochondrial polyP decreased nearly 10-fold as compared with the complete medium. Under phosphate overplus, this level was nearly twofold higher than in the complete medium without preliminary P_i starvation (Table 1). The chain length of acid-soluble polyP of mitochondria was estimated by electrophoresis. Under P_i limitation, their polyP level was too low to be detected by this method. The chain length was <15 phosphate residues. This is close to the value determined by ^{31}P-NMR [24]. Under phosphate overplus, the mitochondrial polyP chains became longer. At the same time, polyP accumulation in mitochondria was not observed during cultivation on ethanol under aeration, even under polyP overplus in the cells [27]. Their content was extremely low (Table 1). Thus, the so-called effect of polyP overplus typical both of yeast cells and of many bacteria [1] was observed in *S. cerevisiae* mitochondria. However, such accumulation of polyP took place under glucose repression unfavorable for the primary mitochondrial function: oxidative phosphorylation. It is known that one of the most important functions of polyP in bacteria is participation in the triggering of cascade reactions for overcoming nutritional and oxidative stresses [3, 8]. The possible protective effect of polyP accumulation in yeast mitochondria under glucose repression needs further study. So far, we can only note that polyP accumulation during the supply of phosphate to the medium after P_i starvation is typical of many microorganisms, including bacteria. Preservation of this ability in mitochondria is interesting for understanding the evolutionary aspects of the overcoming of mitochondrial dysfunction under unfavorable conditions.

Recently, polyP was found in the mitochondria of other eukaryotes. So, the mitochondrial fraction of *Dictyostelium discoideum* contained polyP with chain

length of about 14 phosphate residues [8]. PolyP was also found in the mitochondrial fraction obtained from the cells of insects [29].

One of the important polyP forms in living cells is specific complexes with polyhydroxybutyrate (PHB) and Ca^{2+}, which have been found in the membranes of many organisms and performs a transport channel function [30-33]. These complexes have been found in the membranes of mitochondria of animal cells [30]. They were extracted from rat liver mitochondria and reconstructed into a lipid bilayer [34]. The reconstituted channels showed multiple subconductance levels and were voltage-dependent. This channel is supposed to represent at least some part of the structure of the mitochondrial permeability transition pore [34].

The polyP pool in mitochondria probably consists of two parts; one part is localized directly in the membranes as a polyP–PHB–Ca^{2+} complex, and the other part is present in the intermembrane space and not bound to polyhydroxybutyrate, though its association with cations, including Ca^{2+}, is not improbable.

Thus, mitochondria preserve the evolutionarily ancient forms of ion channels typical of prokaryotes and the ability to accumulate under certain conditions, similar to bacterial cells, higher amount of polyP not related to ion channels.

ENZYMES OF PolyP METABOLISM IN MITOCHONDRIA

The enzymes of polyP metabolism are best studied in *S. cerevisiae*. Its mitochondria possess two exopolyphosphatases (EC 3.6.1.11), splitting P_i from the end of the polyP chain. After subfractionation of *S. cerevisiae* mitochondria, it was revealed that both the membrane preparation and the soluble fraction possessed exopolyphosphatase activities in equal proportion [35]. The membrane-bound activity was partly solubilized by Triton X-100 [35]. Under gel filtration of the solubilized preparation of mitochondrial membranes, this activity was associated with proteins of 76 and 140 kDa, indicating probable oligomerization. The soluble exopolyphosphatase was of 40 kDa. Both activities hydrolyze polyP of different chain length but failed to hydrolyze *p*-nitrophenylphosphate, ATP and other nucleoside triphosphates, and PP_i [21, 35, 36]. The enzyme–substrate affinity was considerably higher with polyP of the longer chain. Both activities were inhibited by heparin and had neutral optimum of pH [35].

Mutants with inactivated genes *PPX1* (http://www.uniprot.org/uniprot/P38698) and *PPN1* (http://www.uniprot.org/uniprot/Q04119), encoding the two known yeast exopolyphosphatases, are currently available [37, 38]. Inactivation of the *PPX1* gene encoding cytosolic exopolyphosphatase had no effect on membrane-bound but decreased soluble exopolyphosphatase activity in mitochondria [39]. The strain with inactivated *PPX1* gene had no soluble 40-kDa exopolyphosphatase in the mitochondrial matrix. Instead, the high molecular weight form of exopolyphosphatase appeared in the soluble fraction [21, 39]. No membrane-bound exopolyphosphatase activity was observed in the mitochondria of the strains with inactivated *PPN1*, while the soluble exopolyphosphatase was represented by 40-kDa enzyme with decreased activity [39]. No exopolyphosphatase activity was detected in the mitochondria of the double mutant with inactivated *PPX1* and *PPN1* [39]. These data suggest that the 40-kDa soluble and membrane-bound exopolyphosphatases of mitochondria are encoded by the *PPX1* and *PPN1* genes, respectively. The presence of high molecular weight soluble exopolyphosphatase in the matrix of *PPX1* mutants is probably due to miss-sorting and aggregation of the *PPN1* product.

Exopolyphosphatase activity was revealed in the mitochondria of embryos of *Rhipicephalus microplus*, an important cattle ectoparasite from South America [29]. The activity was analyzed using $polyP_{15}$ as a substrate. The hydrolysis of polyP was stimulated about two-fold by NADH and strongly inhibited by P_i. Respiratory substrates, pyruvic and succinic acids, stimulated the activity; this effect disappeared upon addition of KCN.

As concerns the synthesis of mitochondrial polyP, the enzymes responsible for this process have not yet been identified. The polyphosphate kinase reaction, during which the terminal ATP residue is transferred to the growing polyP chain, has not been registered in the preparation of yeast mitochondria [26]. Membrane potential-dependent pyrophosphate synthesis revealed in mitochondria [15, 16] might be a first stage of polyP synthesis. Some indirect data also indicate the synthesis of mitochondrial polyP due to this energy: uncouplers (FCCP, dinitrophenol) and ionophores (monensin, nigericin) inhibited polyP accumulation in the mitochondria of *S. cerevisiae* [25].

LOCALIZATION OF PolyP IN YEAST MITOCHONDRIA

Localization of polyP in mitochondria of *S. cerevisiae* was determined by subfractionation of isolated mitochondria by mild osmotic treatment [40, 41]. The fractions of intermembrane space and mitoplasts were obtained [26]. The mitoplasts included a matrix enclosed by the inner membrane and contained an outer membrane attached to the inner mitochondrial membrane due to intermembrane contacts. Distribution of the activities of marker enzymes (adenylate kinase, the enzyme of intermembrane space, and fumarase, the enzyme located in the mitochondrial matrix) in resultant fractions was in agreement with the literature data [40, 41]. The fraction

Table 2. Distribution of acid-soluble polyP and some enzymatic activities between submitochondrial fractions of *S. cerevisiae* (wild strain VKM Y-1173) grown on glucose [26]

Submitochondrial fraction	polyP	Exopolyphosphatase	Adenylate kinase	Fumarase
Intermembrane space	90	5	80	5
Mitoplasts	10	95	20	95

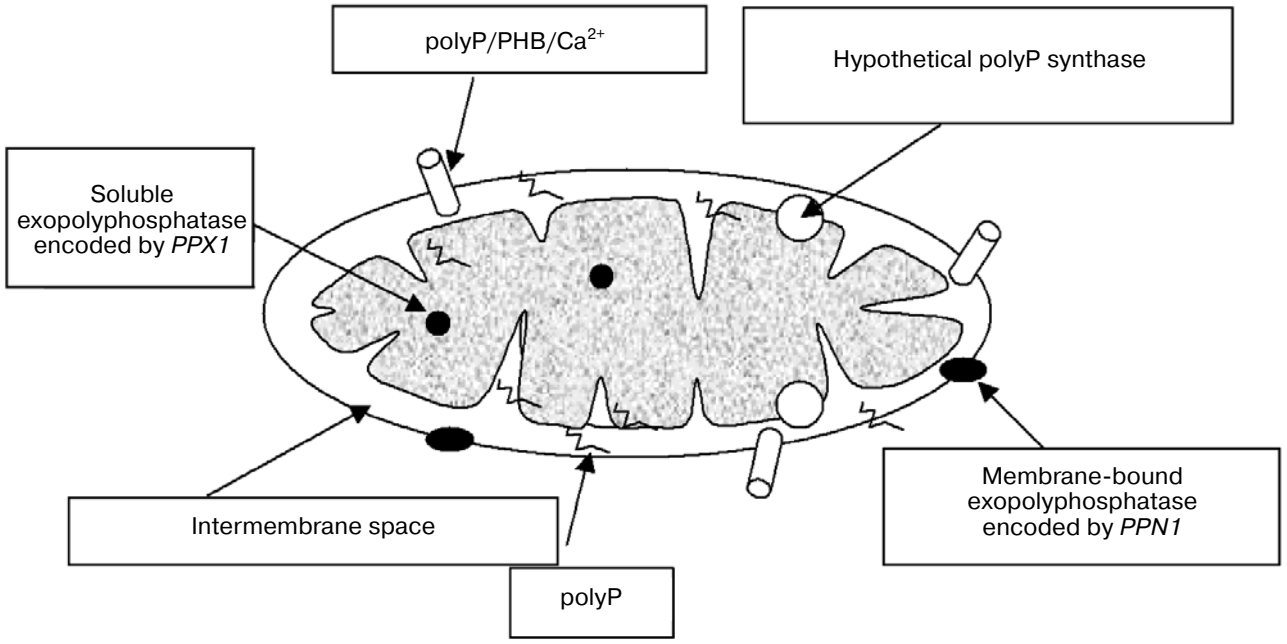
of intermembrane space contained ~90% of total mitochondrial polyP, while ~95% of exopolyphosphatase activity was observed in the mitoplast fraction (Table 2) [25, 26].

The polyP pool in mitochondria probably consists of two parts; one part is localized directly in the membranes as a polyP–PHB–Ca^{2+} complex, and the other part is present in the intermembrane space and not bound to PHB, though its association with cations, including Ca^{2+}, is not improbable.

A hypothetical scheme of localization of polyP and enzymes of its metabolism in yeast mitochondria is presented in the scheme.

EFFECTS OF CARBON SOURCE AND AERATION ON PolyP ACCUMULATION IN *S. cerevisiae*

In view of the fact that energy metabolism and the functional state of mitochondria in *S. cerevisiae* depend on the carbon source and aeration, it would be of interest to consider the peculiarities of polyP accumulation in cells depending on these physiological conditions. The phosphate overplus model was used for this study [42]. Total polyP accumulation was similar under cultivation on glucose and ethanol. However, sufficient difference was observed in the content of separate polyP fractions, i.e. acid-soluble fraction polyP1, salt-soluble fraction polyP2, weakly alkali-soluble fraction polyP3, alkali-soluble fraction polyP4, and polyP5 (Table 3). The maximal levels were independent of the carbon source for fractions polyP2, polyP3, and polyP4. The maximum level of polyP1 was higher on glucose than on ethanol, but the level of polyP5 was higher on ethanol. The average chain length of polyP1 of 15 phosphate residues was minimal, and of polyP5 was maximal (nearly 200 phosphate residues). Thus, the predominant type of bioenergetics substantially influences the accumulation and chain length of polyP in *S. cerevisiae*. The reasons for this difference are still unknown.



Hypothetical scheme of localization of polyP and enzymes of its metabolism in yeast mitochondria

Table 3. Accumulation of different fractions of polyP (μmol P/g of dry biomass) in the cell of *S. cerevisiae* VKM Y-1173 under phosphate overplus (30 min cultivation in phosphate-containing medium after 7 h phosphate starvation) [42]

Cultivation conditions	polyP1		polyP2		polyP3		polyP4		polyP5	
	glucose	ethanol	glucose	ethanol	glucose	ethanol	glucose	ethanol	glucose	ethanol
P_i starvation	18	7.4	9.7	8.5	23.9	14.9	6.4	10.6	7.2	7.1
Phosphate overplus	284	96.8	96.2	63	468	320	51.7	67.1	185	307

POLYPHOSPHATES AND THE FUNCTIONS OF YEAST MITOCHONDRIA

The *S. cerevisiae* strains with inactivated exopolyphosphatase-encoding genes [37, 38] were used in the study of the effect of the inactivations on mitochondrial function [39]. The parent strain and the strain with inactivated *PPX1* gene displayed diauxic growth during glucose consumption. The strains with inactivated *PPN1* gene stopped growing on glucose earlier than the parent strains. They were unable to use non-fermentable carbon sources as substrates and did not grown on ethanol or lactate [39]. The ability of glucose to repress the development of mitochondria in *S. cerevisiae* is well known [43]. In the logarithmic growth phase the parent strain and the strain with inactivated *PPX1* possessed promitochondria, while in the stationary growth phase well developed mitochondria appeared (Table 4). In the strains with inactivated *PPN1* gene even in the stationary growth stage promitochondria with defective respiratory function were present (Table 4).

The inactivation of *PPN1* leads to absence of membrane-bound exopolyphosphatase and defects in polyP metabolism in these organelles [39]. In the mitochondria of the parent strain and the *PPX1* mutant the level of acid-soluble polyP decreased from ~1 μmol P/mg protein in the logarithmic phase to ~0.3 μmol P/mg protein in the stationary phase. Quite on the contrary, the level of acid-soluble polyP in *PPN1* mutants increased from ~0.3 μmol P/mg protein in the logarithmic phase to 0.72 μmol P/mg protein in the stationary phase. Its chain length increased as well. The chain length of mitochondrial polyP in the strains with active *PPN1* decreased during the stationary growth stage, while in the mutants it was increased. As a result, at the stationary stage the promitochondria in the *PPN1* mutant possess polyP of 80-130 phosphate residues, but not the short-chain polyP (~15 phosphate residues) characteristic for the control strain at the stationary growth stage. It is still unclear whether polyP accumulation and elongation could directly cause a breakdown of mitochondrial functions in the *PPN1*

Table 4. Some properties of mitochondrial fractions obtained from *S. cerevisiae* strains grown on glucose, stationary growth phase [39]

Parameter	Strains			
	parent strain	strain with inactivated *PPX1*	strain with inactivated *PPN1*	strain with inactivated *PPX1* and *PPN1*
Respiratory control	2.3	2.1	1	1
P/O ratio	1.5	1.3	0	0
polyP content, μmol/mg protein	0.34	0.27	0.73	0.72
Exopolyphosphatase activities, 10^{-3} U/mg protein				
membrane-bound	97	80	0	0
soluble (40 kDa)	136	0	14	0
soluble high-molecular-weight	0	35	0	0

Note: Strains were kindly provided by Prof. N. Rao and A. Kornberg [37, 38].

mutants, or this process involved some unknown regulatory mechanisms. However, the data on these interrelations point attention to the role of polyP in function and dysfunction of mitochondria. As for the *PPX1* gene, the enzyme encoding it is localized in the matrix where polyP is practically absent. So, it is not surprising that its inactivation had no effect on polyP metabolism. The functions and substrate of this enzyme in mitochondria need further investigation. It should be noted that this enzyme hydrolyzes adenosine 5′-tetraphosphate [44] that probably performs signaling functions.

PARTICIPATION OF PolyP IN THE FUNCTION OF INSECT AND ANIMAL MITOCHONDRIA

In the mitochondria of *Rhipicephalus microplus* embryos, the polyP content changed following the exopolyphosphatase activity during embryogenesis from the first up to the 18th day of development [29]. The exopolyphosphatase activity increased during embryo cellularization and segmentation, from day 5 to 7 of development; at the same time, polyP levels declined. It has been postulated that the polyP of mitochondria are used as an energy supply during embryogenesis of hard ticks [29].

To study the possible role of polyP in animal cells, the authors have designed genetic constructions for reduction of polyP content in the mitochondria of human cell lines [45]. With this purpose, a DNA construct was obtained encoding GFP fused with PPX1 exopolyphosphatase from the yeast. The expression of the recombinant protein in the mitochondria was confirmed by confocal microscopy and by the measurement of exopolyphosphatase activity of the mitochondrial lysate from transfected cells. No detectable endogenous exopolyphosphatase activity was observed in the lysate from the mitochondria of non-transfected cells. The polyP content in the mitochondria of transformed cells was assayed by DAPI fluorescence [45]. DAPI is not a specific dye for polyP, but DAPI–DNA and DAPI–polyP complexes have blue-white and yellow fluorescence, respectively [46]. This allows distinguishing the two complexes. Such DAPI fluorescence significantly reduced under expression of yeast PPX exopolyphosphatase in mitochondria [45]. The functional state of mitochondria under transformation was assessed using the following indicators: mitochondrial membrane potential, the redox state of NADH, and the effects of interventions focused on specific complexes in the electron transport chain. Specific reduction of mitochondrial polyP due to PPX1 expression significantly modulated mitochondrial bioenergetics: reduction of the inner membrane potential and increase in NADH levels were observed. Furthermore, the reduction of polyP levels increases mitochondrial capability to accumulate calcium [45]. Thus, the first data obtained with the cells of mammals demonstrate a substantial role of inorganic polyphosphates in the function of mitochondria. The mechanism of this phenomenon is subject to further investigation. It is not improbable that this role is different in eukaryotes from different stages of evolution.

In conclusion, it should be noted that: (i) mitochondria can contain inorganic polyP, both as membrane structures in association with PHB and Ca^{2+} and as separate forms, and the enzymes of their metabolism; (ii) polyP metabolism in mitochondria depends on the stage of ontogenesis and environmental conditions, in the yeast, polyP accumulation in mitochondria is typical of the conditions of glucose repression, when these organelles are incapable of active function; (iii) genetic modifications affecting the activities of exopolyphosphatases (polyP-hydrolyzing enzymes) lead to significant variations in the functioning of mitochondria; (iv) the question about the mechanisms of polyP synthesis in mitochondria needs further investigation; however, this synthesis is probably not associated with direct utilization of ATP as in the case of polyphosphate kinase reaction in bacteria.

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