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Polyphosphates and Exopolyphosphatases in Cytosol and Mitochondria of *Saccharomyces cerevisiae* during Growth on Glucose or Ethanol under Phosphate Surplus

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Abstract—Content and chain lengths of inorganic polyphosphates (polyP) as well as exopolyphosphatase activities were compared in cytosol and mitochondria of the yeast *Saccharomyces cerevisiae* during growth on glucose or ethanol under phosphate surplus. PolyP metabolism in cytosol and mitochondria was substantially dependent upon the carbon source. Acid-soluble polyP accumulated mainly in cytosol using either glucose or ethanol. The level of the accumulation was lower during growth on ethanol compared to that on glucose. Increase in polyP content in mitochondria was observed during growth on glucose, but not on ethanol. In cytosol the activity of exopolyphosphatase PPN1 was increased and the activity of exopolyphosphatase PPX1 was decreased independently of the carbon source under phosphate surplus conditions. Growth on ethanol caused exopolyphosphatase PPN1 to appear in the soluble mitochondrial fraction, while during growth on glucose only exopolyphosphatase PPX1 was present in this fraction.

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Functions of inorganic polyphosphates (polyP), linear polymers of orthophosphoric acid, in microorganisms are tightly connected to energy metabolism [1-3]. In bacteria these polymers are involved in regulation of intracelullar concentration of ATP and other nucleoside triphosphates due to the presence of polyphosphate kinase and polyphosphate:AMP-phosphotransferase enzymes; polyphosphate glucokinase and polyphosphate:NADP-phosphotransferase utilize polyP instead of ATP in transphosphorylation reactions [2, 3]. The involvement of polyP in energy metabolism is less investigated in eucaryotic cells. The most important polyP metabolizing enzymes are exopolyphosphatases (polyphosphate phosphohydrolase, EC 3.6.1.11) localized in different cell compartments [4]. Two genes encoding polyP-metabolizing enzymes have been identified in *Saccharomyces cerevisiae* [5-7]. The *PPX1* gene encodes exopolyphosphatase with molecular weight of approximately 40 kD localized in cytosol, cell envelope, and soluble mitochondrial fraction [4, 5, 8]. The *PPN1* gene encodes high molecular weight cytosolic exopolyphosphatase, which is different from the PPX1 enzyme in a number of physicochemical properties and expression conditions [9]. Products of this gene are localized in other cell compartments too [10].

Since polyP are macroergic compounds, it was interesting to compare their metabolism in yeasts under conditions of glycolysis (growth on glucose) or oxidative phosphorylation (growth on ethanol) with special attention to cytosol and mitochondria. During *S. cerevisiae* growth on glucose, i.e. under glucose repression characterized by the prevalence of promitochondria [11], polyP and exopolyphosphatases occur in cytosol and mitochondria [8, 12, 13]. However, there are no such data for the oxidative phosphorylation conditions.

The aim of the present study was to investigate the influence of the carbon source on the accumulation of polyP and on the activities of the two exopolyphosphatases encoded by the *PPX1* and *PPN1* genes in mitochondria and cytosol of *S. cerevisiae* under phosphate surplus.

Abbreviations: polyP) polyphosphates.

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MATERIALS AND METHODS

Saccharomyces cerevisiae strain VKM Y-1173 was grown under phosphate surplus conditions as described earlier [8]. Cells were grown to stationary phase (24 h) on phosphate-deficient Reader (–P) medium with glucose and 1.3 mM P_i, separated by centrifugation at 5000*g* for 10 min, washed with distilled water, and transferred in Reader (+P) medium containing 18.3 mM P_i and 2% of glucose or ethanol and were cultivated for 20 h. The medium volumes in flasks were 200 and 50 ml, shaking velocities were 120 and 220 rpm during growth on glucose or ethanol, respectively.

Spheroplasts and cytosol fraction were obtained as previously described [8]. The pellet obtained after separation of cytosol fraction by centrifugation was considered as a total fraction of organelles and membranes and was used for the extraction of acid-soluble polyP.

The known differential centrifugation scheme was applied for purification of mitochondria [14]. Spheroplasts washed with 1.2 M sorbitol were harvested at 0°C and gentle stirring in 20 mM Tris-HCl (pH 6.8) in presence of 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA, and 4 mM $MgSO_4$. Concanavalin A (0.05%) was added to the buffer to remove plasmatic membranes during low-speed centrifugation. Spheroplast harvesting was monitored by light microscopy. After the described treatment, the suspension was rapidly added the an equal volume of the same buffer containing 1.2 M sorbitol with stirring and was low-speed centrifuged at 2500*g* for 10 min. The supernatant was centrifuged at 10,000*g* for 10 min to precipitate the mitochondria. The pellet was homogenized in the same buffer containing 0.6 M sorbitol using a Teflon pestle and centrifuged at 2500*g* for 10 min, and then mitochondria were pelleted at 10,000*g* for 10 min.

To obtain soluble mitochondrial fraction the mitochondria were resuspended in the same buffer without sorbitol, sonicated 5 times for 20 sec at 0°C using an MSE ultrasonic disintegrator (Braun, USA), and centrifuged at 12,000*g* for 60 min. The supernatant constituted the soluble mitochondrial fraction.

The mitochondrial preparation did not contain plasma membranes, as confirmed by the absence of vanadate-sensitive ATPase activity, a marker protein of plasma membranes [15], and vacuoles, since no activity of the vacuole marker enzyme, nitrate-sensitive ATPase [16], was detected. Specific ATPase activity in the mitochondrial fraction at pH 7.2 was equal to 1.16 U/mg protein and was inhibited by 70% by 5 mM NaN_3.

Heparin (20 mg/ml), an exopolyphosphatase inhibitor [17], was added in buffers to obtain subcellular fractions for the subsequent analysis of polyP. Acid-soluble polyP from spheroplasts and subcellular fractions were extracted with 0.5 N $HClO_4$ at 0°C [18]. Acid-soluble polyP content was determined using labile phosphorus [18]. PolyP was subjected to electrophoresis on 20% polyacrylamide gel in presence of 7 M urea and was stained in the gel with Toluidine Blue [19].

Exopolyphosphatase was separated by gel filtration on an FPLC system (Pharmacia, Sweden) with Superose-6 column equilibrated with 20 mM Tris-HCl buffer (pH 7.2) containing 2 mM $MgSO_4$, 0.1 M KCl, and 0.1% Triton X-100. The following proteins were used as standards: ferritin (440 kD), β-amylase (200 kD), alcohol dehydrogenase (150 kD), bovine serum albumin (66 kD), carboanhydrase (29 kD), and cytochrome *c* (12.4 kD) (Sigma, USA). PPX1 and PPN1 activities were assessed using the total activity of fractions with molecular weight of ~40 and ~440 kD [8]. Taking the total exopolyphosphatase activity as 100%, PPX1 and PPN1 contribution was calculated using the activities of fractions of corresponding molecular weight.

Exopolyphosphatase activity was measured as a rate of P_i formation [8]. The reaction medium contained 50 mM Tris-HCl, pH 7.2, 2.5 mM $MgSO_4$, and 0.01 mM polyP with average chain length of 208 phosphate residues. PolyP (Monsanto, USA) was purified free from P_i and PP_i [20] before use. Protein concentration was determined using bovine serum albumin as a standard [21]. One activity unit corresponds to the quantity of enzyme forming 1 μmol of P_i per minute.

RESULTS AND DISCUSSION

It is known that both cytosol and soluble mitochondrial fraction of *S. cerevisiae* contains exopolyphosphatases PPX1 and PPN1 [8, 10], and its ratio is dependent upon the growth conditions. Enzyme encoded by gene *PPN1* occurs in these compartments during inactivation of the *PPX1* gene [10], as well as in cytosol under special growth conditions when cells transfer from stationary phase to a new budding in the presence of glucose and excess of P_i [8, 9, 18]. Thus, during re-inoculation of *S. cerevisiae* in its late logarithmic phase from (–P) glucose-containing medium to (+P) glucose-containing medium and subsequent growth to stationary phase, cytosol PPX1 activity decreased 10-fold, while PPN1 exopolyphosphatase activity increased 8-fold [8, 18]. At the same time, the soluble mitochondrial fraction still contained only the PPX1 enzyme [22].

In the present study, we determined the activity of exopolyphosphatases PPX1 and PPN1 in cytosol and soluble mitochondrial fraction under phosphate surplus conditions using ethanol as the carbon source compared to glucose. After re-inoculation of phosphate-starved cells in (+P) medium with ethanol, PPX1 enzyme was not found in cytosol during stationary growth phase, and overall exopolyphosphatase activity was due to the presence of PPN1 (Table 1). Under the same conditions in glucose-containing medium, the PPX1 activity was 12%

Table 1. Exopolyphosphatase activities in cytosol and soluble mitochondrial fraction of *S. cerevisiae* during growth on ethanol or glucose to stationary phase

Growth conditions	Cytosol		Soluble mitochondrial fraction	
	exopoly-phosphatase activity, mU/mg protein	PPN1 contribution, %	exopoly-phosphatase activity, mU/mg protein	PPN1 contribution, %
Phosphate-limited, glucose-containing medium	110	6	150	0
Phosphate surplus				
glucose-containing medium	80	88	70	0
ethanol-containing medium	230	100	380	55

Table 2. P_i and acid-soluble polyP content (μmol P per g dry biomass of starting culture) in spheroplasts, cytosol, and in the total fraction of membranes and organelles under phosphate surplus conditions

Carbon source	Spheroplasts		Cytosol		Total membrane and organelle fraction	
	P_i	polyP	P_i	polyP	P_i	polyP
Glucose	61	540	190	290	32	74
Ethanol	35	150	74	58	16	36

Note: Cells were grown to stationary phase on phosphate-deficient medium and re-inoculated in phosphate-containing medium as described in "Materials and Methods".

of the total exopolyphosphatase activity of the cytosol. Under surplus conditions, the PPN1 enzyme did not appear in soluble mitochondrial fraction when glucose was used as the carbon source. However, during growth on ethanol this compartment contained PPN1, whose contribution to the total exopolyphosphatase activity was 55% (Table 1).

Therefore, independently of the carbon source, under phosphate surplus conditions exopolyphosphatase PPN1 is a prevalent enzyme in the cytosol of *S. cerevisiae*. Furthermore, during growth on ethanol, unlike that on glucose, this enzyme begins to dominate in soluble mitochondrial fraction. This indicates that specific pathways of the regulation of expression of these proteins exist that together with the P_i content in the medium can be controlled not only by the presence of glucose but also by that of ethanol.

PolyP of cytosol and mitochondria comprise mainly the acid-soluble fraction [10, 22]. That is why only this fraction was analyzed. During yeast growth on (−P) glucose-containing medium to stationary phase, the acid-soluble polyP content in spheroplasts and cytosol did not exceed 10-11 μmol P_i per g of dry biomass of the initial culture. Under phosphate surplus conditions using both carbon sources, a significant increase in acid-soluble polyP content was observed in spheroplasts and cytosol, but the effect was most prominent during growth on glucose (Table 2). The polyP content in cytosol was more than 54 and 39% of that in spheroplasts during growth on glucose or ethanol, respectively. Despite the addition of heparin, an effective exopolyphosphatase inhibitor [4, 17], into solutions during purification of subcellular fractions, it is likely that partial degradation of polyP took place during cytosol separation, indicated by higher content of P_i in it as compared to that of spheroplasts (Table 2). The total content of P_i and polyP in cytosol was 80 and 70% from that in spheroplasts for growth on glucose or ethanol, respectively. These data, showing the primary localization of polyP to be in cytosol, are in good agreement with previously obtained results for other *S. cerevisiae* strains [2, 10]. The content of acid-soluble polyP in total fraction of membranes and organelles was higher during growth on glucose compared to that on ethanol (Table 2).

In mitochondria, polyP content was substantially higher under phosphate surplus conditions and growth on glucose compared to growth on (+P) medium without surplus (0.24 μmol P per mg protein) [22], while there

was no such increase when ethanol was used as the carbon source (Table 3). These data are in agreement with previous results for such an oxidizable substrate as lactate, where polyP content in mitochondria was much lower than that during growth on glucose [23]. Therefore, polyP content is lower during growth on ethanol compared to that on glucose. This conforms to the increase in PPN1 exopolyphosphatase activity (Table 1).

The figure shows cytosolic and mitochondrial polyP electrophoresis under phosphate surplus. In cytosol, the average chain length of polyP did not depend upon the carbon source used. The chain length of the major part of polyP corresponded to commercial polyP formulation with average chain length of 45 (Sigma, USA). In mitochondria, during growth on glucose the major part of polyP had chain length identical to that of the formulation with the average chain length of 25 (Sigma). For samples grown on ethanol, it was not possible to identify mitochondrial polyP by means of electrophoresis or to obtain its barium salt precipitate, probably due to low concentration and chain length.

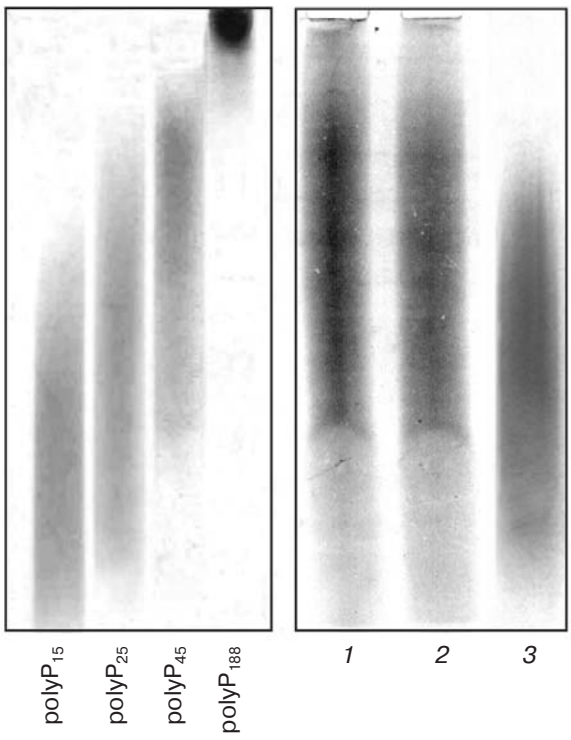
PolyP content in cytosol and mitochondria of *S. cerevisiae* is dependent upon the carbon source under surplus conditions. The use of oxidizable substrate (ethanol) led to the decrease of polyP accumulation in both compartments. The polyP level in mitochondria during growth on ethanol did not reach the level of polyP even during growth on glucose in presence of P_i, but without surplus. Therefore, when using ethanol as a carbon source the contribution of mitochondria in polyP metabolism can be considered as insignificant.

The data indicate that not only glucose but also ethanol can serve as a signal for increase in exopolyphosphatase PPN1 expression under surplus. Furthermore, during growth on ethanol, exopolyphosphatase expression profile changes both in cytosol, and in soluble mitochondrial fraction. The appearance of PPN1 enzyme in soluble mitochondrial fraction was previously observed in a *PPX1* mutant [10].

During growth on oxidizable substrate polyP content decrease, and this decrease is more pronounced in mitochondrial than in cytosol. It is likely that the presence of polyP in mitochondria during growth on glucose is a characteristic of adverse conditions for the functioning of these organelles connected with catabolic repression.

Table 3. P_i and acid-soluble polyP (μmol P per mg of protein) in mitochondria

Growth conditions	P_i	polyP
Phosphate limited, glucose-containing medium	0.02	0.006
Phosphate surplus glucose-containing medium	0.13	0.85
ethanol-containing medium	0.22	0.10



Electrophoresis of acid-soluble polyP from cytosol and mitochondria in 20% polyacrylamide gel containing 7 M urea (Toluidine Blue staining): *1*) cytosol, growth on ethanol under surplus; *2*) cytosol, growth on glucose under surplus; *3*) mitochondria, growth on glucose under surplus. PolyP with average chain length of 15, 25, 45 (Sigma), and 188 (Monsanto, USA) phosphate residues were used

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