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EXPERIMENTAL ARTICLES

Inactivation of the *PPN1* Gene Exerts Different Effects on the Metabolism of Inorganic Polyphosphates in the Cytosol and the Vacuoles of the Yeast *Saccharomyces cerevisiae*

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Abstract—Inactivation of the *PPN1* gene, encoding one of the enzymes involved in polyphosphate metabolism in the yeast *Saccharomyces cerevisiae*, was found to decrease exopolyphosphatase activity in the cytosol and vacuoles. This effect was more pronounced in the stationary growth phase than in the phase of active growth. The gene inactivation resulted in elimination of a 440-kDa exopolyphosphatase in the vacuoles but did not influence a previously unknown vacuolar exopolyphosphatase with a molecular mass of >1000 kDa, which differed from the former enzyme in the requirement for bivalent cations and sensitivity to heparin. Inactivation of the *PPN1* gene did not influence the level of polyphosphates in the cytosol but increased it more than twofold in the vacuoles. In this case, the polyphosphate chain length in the cytosol increased from 10–15 to 130 phosphate residues both in the stationary and active growth phases. In the vacuoles, the polyphosphate length increased only in the stationary growth phase. A conclusion can be made that the *PPN1* gene product has different effects on polyphosphate metabolism in the cytosol and the vacuoles.

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Inorganic polyphosphates (polyP) and enzymes involved in their metabolism are vitally important to the yeast cell [1–3]. The *PPX1* and *PPN1* genes presently found in yeasts code for, respectively, a 40-kDa exopolyphosphatase (polyphosphate phosphohydrolase, EC 3.6.1.11) [3, 4] and polyphosphate depolymerase (EC 3.6.1.10), an enzyme that possesses either endopolyphosphatase [5] or exopolyphosphatase [6] activity.

It is known that the content of polyP molecules and their length and exopolyphosphatase activity in yeast cells vary considerably depending on the growth phase [7, 8]. The proteins responsible for these changes mostly remain unknown. Researchers have shown that inactivation of the *PPN1* (*PHM5*) gene increases the average chain length of polyphosphates in *Saccharomyces cerevisiae* cells [5, 9]. Polyphosphates and exopolyphosphatases may localize in several cell compartments. For this reason, it was of interest to evaluate the effect of the *PPN1* gene inactivation on polyphosphate metabolism in the cytosol and the vacuoles, which show great difference in this metabolism [10].

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It is assumed that the *PPN1* gene product is localized in the yeast vacuoles [5, 6], although there is no direct evidence for this. According to recent studies, the *PPN1* gene product considerably influences exopolyphosphatase activity in the cytosol of *S. cerevisiae*. Inactivation of this gene suppresses the production of both the PPX1 protein and a separate high-molecular-mass exopolyphosphatase (M_r >1000 kDa), which is a product of another gene [11].

The aim of this work was to study the effect of inactivation of the *PPN1* gene on exopolyphosphatase activity and to estimate the level and the length of cytosolic and vacuolar polyphosphates in the *S. cerevisiae* cells occurring in different growth phases.

MATERIALS AND METHODS

Strains and cultivation conditions. The parent strain *Saccharomyces cerevisiae* CRY and its mutant CRN (with the inactivated *PPN1* gene) were kindly provided by A. Kornberg and N. Rao from Stanford University, United States. The strains were grown aerobically at 30°C on YPD medium, containing 1% yeast extract, 2% peptone, and 2% glucose [5]. This medium (250 ml) was inoculated with cells grown on YPD agar

plates. To obtain synchronously budding cells of the CRY and CRN strains, cells of these strains grown to the stationary growth phase were harvested by centrifugation at 5000 *g* for 10 min, washed with cold sterile water, and suspended in fresh YPD medium in a proportion of 2 g wet cells per 250 ml of the medium, which corresponded to a culture turbidity at 600 nm equal to approximately 5. After 5 h of cultivation, when the culture occurred in the exponential growth phase, cells were harvested and washed as described above. A comparative analysis of the 24-h-old stationary-phase cells and partially synchronized 5-h-old exponential-phase cells was carried out for both parent and mutant strains [11].

Preparation of spheroplasts. Cells (1 g wet biomass) were suspended in medium A (8 ml), containing 0.8 M mannitol, 1.5% of lyophilized snail gut juice (*Helix pomatia*), 50 mM dithiothreitol (DTT), and 0.14 M sodium citrate (pH 6.7). The suspension was incubated at 30°C for 70 min in order to induce the formation of spheroplasts. Then, the spheroplasts were precipitated and washed in medium A lacking the juice and DTT.

Preparation of cytosol. Cytosolic fraction was prepared by the method of osmotic lysis of spheroplasts in 0.1 M sorbitol, with a subsequent centrifugation of the preparation at 100000 *g* for 3 h [11]. The purity of the cytosol (i.e., the absence of contaminating organelles, such as plasma membranes, mitochondria, and vacuoles) was confirmed by the absence of ATPase activity sensitive to *ortho*-vanadate (an inhibitor of the ATPase of plasma membranes), azide and oligomycin (inhibitors of mitochondrial ATPase), as well as to nitrate (an inhibitor of vacuolar ATPase).

Preparation of vacuoles and vacuolar sap. Vacuoles were prepared as described earlier [12] and examined for purity by phase-contrast microscopy and the absence of marker enzymes of other cell organelles. Analysis showed that the ATPase activity of the vacuoles was inhibited by 50 mM nitrate (an inhibitor of vacuolar ATPase) and was not inhibited by *ortho*-vanadate and azide (inhibitors of plasmalemma and mitochondrial ATPases, respectively).

Vacuolar sap was prepared by freezing–thawing vacuoles in 10 mM Tris–HCl buffer (pH 7.2) containing 10% glycerol and 0.5 mM phenylmethylsulfonyl fluoride (an inhibitor of proteases) [13]. The exopolyphosphatase activity of the vacuolar sap was 3 to 4 times that of the tonoplast, although these enzyme activities virtually did not differ in their properties.

Gel filtration. The vacuolar sap was subjected to gel filtration on an FPLC Superose 6 column (Pharmacia, Sweden). The elution buffer was 20 mM Tris–HCl (pH 7.2) with 100 mM KCl. In some experiments, the buffer was supplemented with 0.1% Triton X-100. The flow rate was 1 ml/min. The molecular mass marker kit (Pharmacia) included thyroglobulin (669 kDa), ferritin (440 kDa), β-amylase (200 kDa), aldolase (160 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (13 kDa).

Extraction and analysis of polyphosphates. Acid-soluble fractions of polyphosphates were prepared by treating the preparations of vacuoles or cytosol with 0.5 N $HClO_4$ (in fact, the preparations were mixed with equal volumes of 1 N $HClO_4$) at 0°C for 15 min with continuous stirring. Then the suspension was centrifuged at 5000 *g* for 20 min. The procedure was repeated, and the two supernatants were pooled. To remove nucleotides from the pooled supernatant, it was supplemented with activated charcoal Norit A and incubated in the cold for 30 min [7].

The content of labile phosphorus in the preparations of acid-soluble polyphosphates was determined from the difference in the orthophosphate contents of these preparations before and after their hydrolysis in 1 N HCl at 100°C for 10 min [7, 8].

Electrophoresis of polyphosphates. The pH of the preparation of acid-soluble polyphosphates was adjusted to 4.5 with NaOH, and the polyphosphates were precipitated by adding a saturated solution of $Ba(NO_3)_2$. The suspension was centrifuged at 5000 *g* for 20 min. The precipitated barium salt of polyphosphates was converted to a soluble form by treating it with Dowex 50 NX 8 ion-exchange resin in NH_4^+ form and then adding 10–20 μl of distilled water. The preparation was subjected to electrophoresis in 20% PAAG containing 7 M urea. The separated polyphosphates were stained with toluidine blue [14]. Polyphosphates with chain lengths of 15, 25, 45 (Sigma, United States), and 188 (Monsanto, United States) phosphate residues were used as the standards.

Phosphohydrolase assay. Exopolyphosphatase activity was determined from the rate of inorganic phosphate (P_i) formation at 30°C for 20–30 min in 1 ml of a reaction mixture containing 50 mM Tris–HCl (pH 7.2), 0.1 mM $CoSO_4$, and 9.6 μM $polyP_{208}$. $PolyP_{208}$ was selected for analysis since all the known exopolyphosphatases are most active with this particular substrate [3].

In the experiments on acid-soluble polyphosphates, spheroplasts were lysed in the presence of 4 mg/ml heparin (an inhibitor of exopolyphosphatases) and 20 mM EDTA (another potent inhibitor of enzymes) [3]. Heparin and EDTA were added to all the solutions used for the isolation of vacuoles and cytosol. However, heparin and EDTA were not added when exopolyphosphatase activity was measured.

ATPase was assayed in 50 mM Tris–HCl buffer (pH 7.2) containing 1 mM ATP and 2.5 mM $MgSO_4$.

Other procedures. Protein was quantified by a modification of the Lowry method [10] with BSA as the standard.

Table 1. The levels of acid-soluble polyphosphates (in μmol/mg protein) and exopolyphosphatase activity (in mU/mg protein, given in parentheses) in the cytosol and vacuoles of the parent (CRY) and mutant (CRN) strains of *S. cerevisiae*

Strain	Cytosol		Vacuoles	
	Stationary growth phase	Active growth phase	Stationary growth phase	Active growth phase
Parent CRY	4.6 (130)	1.6 (180)	10.6 (375)	2.1 (250)
Mutant CRN with the inactivated *PPN1* gene	3.4 (30)	1.3 (70)	26.6 (20)	5.1 (125)

α-Mannosidase, succinate dehydrogenase, and glucose-6-phosphate dehydrogenase were assayed as described earlier [10].

The results presented in the paper are the means of triplicate measurements. Experimental errors did not exceed 10%.

RESULTS

To study the effect of the *PPN1* gene inactivation on polyphosphate metabolism in the cytosol and vacuoles of the yeast *S. cerevisiae*, we compared exopolyphosphatase activity, the content of polyphosphates, and their length in the stationary growth phase, when the relative number of budding cells in the culture was 19–30%, and in the phase of active growth, when the percentage of budding cells reached 90% [11]. The selection of the cytosol and vacuoles for study was motivated by the high level of cytosolic polyphosphates in the stationary-phase yeast cells (more than 90% of the total) and by the highest relative content (with respect to proteins) of polyphosphates in the vacuoles. Of great importance for experimental unambiguity was the fact that inactivation of the *PPN1* gene influenced neither the morphology of vacuoles nor their size and number in cells (data not shown). The yield of vacuoles from the parent CRY and mutant CRN cells was nearly the same (0.45 and 0.47 mg protein per g wet biomass). The activity of vacuolar ATPase in the two strains was also very close (approximately 350 mU/mg protein).

The level of polyphosphates in the cytosol and vacuoles of both strains depended on the growth phase (Table 1), being considerably lower in the stationary than in the active growth phase. Such a distribution of polyphosphates over the cytosol and vacuoles resembled their distribution in the whole cells [5]. Inactivation of the *PPN1* gene resulted in a more than twofold increase in the level of vacuolar polyphosphates in both growth phases, while the level of cytosolic polyphosphates did not notably change (Table 1).

Inactivation of the *PPN1* gene differently influenced the average chain length of cytosolic and vacuolar polyphosphates. In the parent strain, the level of cytosolic short-chain polyphosphates was considerably higher in the active than in the stationary growth phase (Figs. 1a, 1b). Similar data were obtained earlier for the distribution of acid-soluble polyphosphates in the whole *S. cerevisiae* VKM Y-1173 cells [7]. As expected, inactivation of the *PPN1* gene resulted in an increase in the average chain length of cytosolic polyphosphates in both the active and stationary growth phases (from 10–15 to 15–130 phosphate residues) (Figs. 1c, 1d).

In the active growth phase of both strains, the length of vacuolar polyphosphates was the same (Figs. 1f, 1h). However, inactivation of the *PPN1* gene increased the average chain length of vacuolar polyphosphates in the stationary-phase cells from 10–25 to 20–130 phosphate residues (Figs. 1e, 1g).

Inactivation of the *PPN1* gene reduced cytosolic exopolyphosphatase activity, the decrease being most pronounced in the stationary growth phase (Table 1). According to our earlier observations, inactivation of the *PPN1* gene results, first, in the absence of the 1000-kDa high-molecular-mass cytosolic exopolyphosphatase, which is active in the exponential growth phase of the yeast *S. cerevisiae* [8, 11] and, second, in a decrease in the activity of the major cytosolic exopolyphosphatase, which is encoded by the *PPX1* gene [11].

Close results were obtained for vacuolar exopolyphosphatase activity, except that this activity considerably diminished in the active growth phase of the parent strain (Table 1).

According to the previous study [11], inactivation of the *PPN1* gene substantially influences the range of cytosolic exopolyphosphatases. In the present study, such experiments were carried out on the vacuoles isolated from exponential- phase cells, since exopolyphosphatase activity in the vacuoles of stationary-phase mutant cells was very low (approximately 15 times lower than in the parent strain). The experiments showed that the vacuolar exopolyphosphatases of the parent and mutant strains considerably differed in properties. First, Co^{2+} ions increased the activity of vacuolar exopolyphosphatase tenfold in the mutant cells, but only twofold in the parent cells (Table 2). Mg^{2+} ions increased vacuolar exopolyphosphatase activity only in mutant cells (Table 2). Heparin (a competitive inhibitor of all known exopolyphosphatases [3]) at a concentration of 1 μg/ml inhibited vacuolar exopolyphosphatase activity in the parent and mutant strains by 40 and 75%, respectively.

The suggestion that the vacuolar exopolyphosphatases of the parent and mutant strains are different

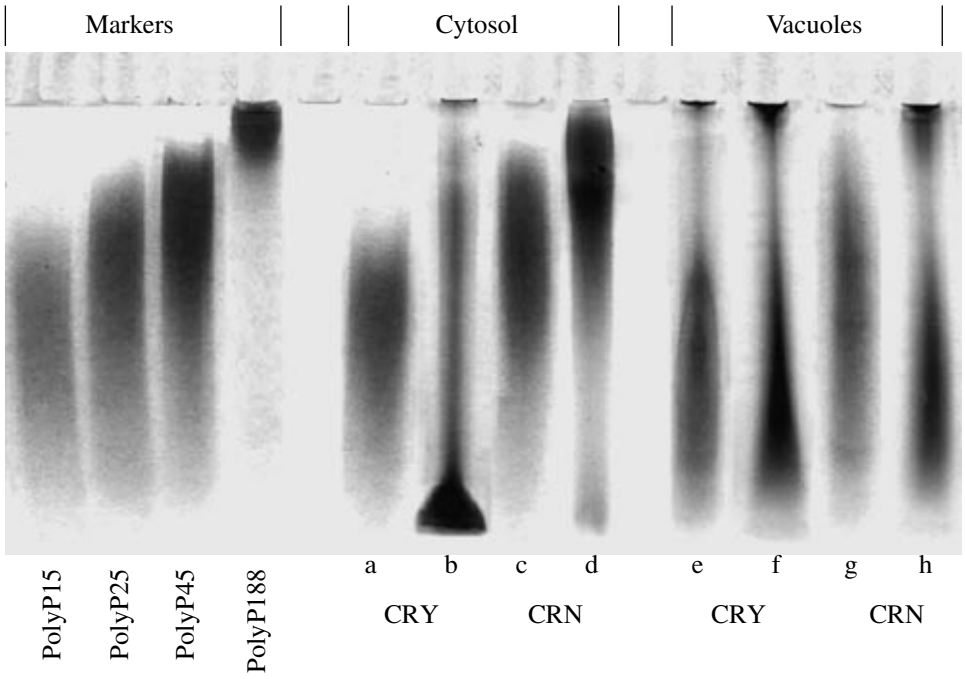

Fig. 1. Electrophoresis of the acid-soluble polyphosphates of the vacuoles and cytosol isolated from the parent (CRY) and mutant (CRN) strains of *S. cerevisiae* grown to the stationary (a, c, e, g) and exponential (b, d, f, h) growth phases.

was confirmed experimentally when the vacuolar sap of the parent and mutant strains was subjected to gel filtration with or without 0.1% Triton X-100 (Fig. 2).

In the absence of the detergent in the elution buffer, the vacuolar exopolyphosphatase activity of the parent strain showed two major peaks, with molecular masses of approximately 440 and 1000 kDa (Fig. 2, Table 3). The product with the higher molecular mass might represent an enzyme aggregate or an enzyme–polyphosphate complex, as is shown for polyphosphate glucokinase [15] and high-molecular-mass cytosolic exopolyphosphatase [16].

Table 2. The effect of various cations and heparin on the exopolyphosphatase activity of the vacuoles isolated from the exponential-phase cells of the parent (CRY) and mutant (CRN) strains of *S. cerevisiae*

Conditions for enzyme assay	Exopolyphosphatase activity, mU/mg protein	
	Parent strain CRY	Mutant strain CRN with the inactivated *PPN1* gene
Control (without additions)	140	12
0.1 mM Co^{2+}	250	125
2.5 mM Mg^{2+}	140	63
0.1 mM Co^{2+}, 1 μg/ml heparin	150	30

The addition of Triton X-100 to the elution buffer did not influence the position, width, or the total exopolyphosphatase activity of the 440-kDa protein peak, although it led to the elimination of the 1000-kDa peak (Fig. 2). Consequently, the detergent destroyed the high-molecular-mass complex, leaving the low-molecular-mass product unchanged.

In the mutant CRN strain, vacuolar sap showed only one peak of exopolyphosphatase activity, with a molecular mass of >1000 kDa (Fig. 2, Table 3). The addition of Triton X-100 to the elution buffer led to the absence of this peak and to a 50% decrease in the enzyme activity.

These data confirm the suggestion of Wurst et al. that the vacuoles of *S. cerevisiae* contain two different exopolyphosphatases [4]. These exopolyphosphatases are probably encoded by two different genes, since inactivation of the *PPN1* gene only leads to the absence of the 440-kDa exopolyphosphatase, which is thus the likely product of the *PPN1* gene.

DISCUSSION

The comprehensive analysis of exopolyphosphatase activity in different compartments of the yeast cells grown under different conditions to different growth phases shows that there are more forms of exopolyphosphatase than was anticipated earlier [17]. In addition to the already reported high-molecular-mass cytosolic exopolyphosphatase [3, 8, 11], we present here

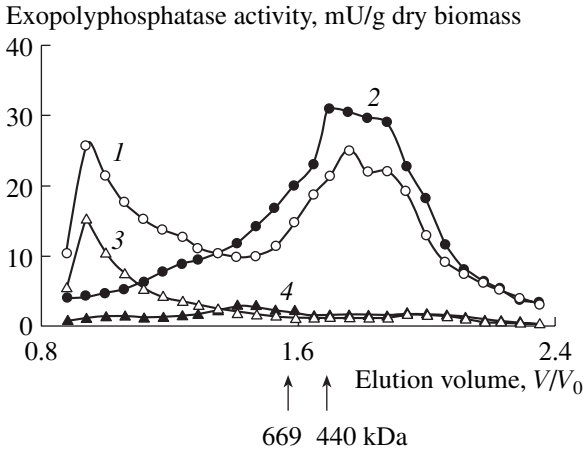

Fig. 2. Gel filtration of vacuolar saps on FPLC Superose 6 column: sap from the parent CRY strain subjected to gel filtration in the presence (*2*) and absence (*1*) of Triton X-100, sap from the mutant CRN strain subjected to gel filtration in the presence (*4*) and absence (*3*) of Triton X-100.

experimental evidence for the existence of a new vacuolar exopolyphosphatase.

The *PPN1* gene (known also as the *PHM5* gene) attracts researchers' interest due to its ability to exert a notable effect on the metabolism of polyphosphates in yeasts. The mutation of this gene gives rise to a phenotype with very long polyphosphate chains but does not influence the level of polyphosphates in the whole cells [8]. Inactivation of the *PPN1* gene reduces the survival of mutant yeasts in the stationary growth phase [5], which can be accounted for by the inability of such mutants to utilize nonfermentable carbon sources because of the impairment of polyphosphate metabolism and lesions in mitochondria [18]. It is believed that the amino acid sequences encoded by this gene may have domains that specify the vacuolar, cytoplasmic, and membrane localization of the gene product [19]. This idea is confirmed by the experiments performed in this and earlier works. Indeed, inactivation of the *PPN1* gene eliminates the high-molecular-mass cytosolic exopolyphosphatase [11], the membrane-bound mitochondrial exopolyphosphatase [18], and one of the vacuolar exopolyphosphatases (this work). All three of these enzymes, in spite of their different properties, may be encoded by one gene. A comparison of the effect of the *PPN1* gene inactivation on the exopolyphosphatases of the cytosol and vacuoles suggests that the high-molecular-mass complexes of these cell compartments are different. Indeed, inactivation of the *PPN1* gene eliminates the complex in the cytosol, leaving it unchanged in the vacuoles.

Table 3. Characteristics of exopolyphosphatase activity in the vacuolar sap prepared from the exponential-phase cells of the parent (CRY) and mutant (CRN) strains of *S. cerevisiae*

Strain	Molecular mass	
	>1000 kDa	~440 kDa
Parent CRY	75	175
Mutant CRN with the inactivated *PPN1* gene	125	0

Note: Fractions with the indicated molecular masses were obtained by gel filtration in the absence of Triton X-100 in the elution buffer. For other conditions of gel filtration, see the Materials and Methods section.

According to the data of Sethuraman et al. [5], the *PPN1* gene product possesses endopolyphosphatase activity, although more recent data show that this product possesses exopolyphosphatase activity, cleaving terminal orthophosphate from the polyphosphate chain [6].

It should be noted that the effect of the *PPN1* gene inactivation on exopolyphosphatase activity can be explained in a different manner. In particular, inactivation of the *PPN1* gene may affect the expression of the other *PPX1* gene through a previously unknown regulatory mechanism [11]. To decide between these possibilities, one must know the amino acid sequences of these enzymes.

To conclude, this work provides experimental evidence that the metabolism of polyphosphates in the cytosol and vacuoles of yeasts is different. Inactivation of the *PPN1* gene does not influence the polyphosphate content in the cytosol but augments it more than twofold in the vacuoles. In this case, the polyphosphate length in the cytosol increases both in the stationary growth phase and in the phase of active growth. In the vacuoles, the polyphosphate length only increases in the stationary growth phase. These data suggest that the *PPN1* gene considerably influences the range of exopolyphosphatases in cells and that the *PPN1* gene product specifies the length of polyphosphate chains in different compartments of the *S. cerevisiae* cells.

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