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EXPERIMENTAL ARTICLES

The Patterns of Utilization and Accumulation of Polyphosphates in the Cytosol of the Yeast *Saccharomyces cerevisiae* under Inactivation of Exopolyphosphatase Genes *PPX1* and *PPN1*

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Abstract—The effect of inactivation of the *PPX1* and *PPN1* genes encoding the yeast exopolyphosphatases on the activities of these enzymes and polyphosphate content in the cytosol of *Saccharomyces cerevisiae* was studied under P_i deficit and P_i excess in the cultivation medium. Under P_i deficit, exopolyphosphatase activity in strain CRN (with inactivated *PPN1* gene) and in the parent strain CRY increased 3- and 1.5-fold, respectively. In the strain CRX (with inactivated *PPX1* gene), exopolyphosphatase activity did not change under P_i deficit. Transfer from P_i-deficient to P_i-rich medium was accompanied by an ~1.7-fold increase of exopolyphosphatase activities in the cytosol preparations of strains CRY, CRX, and CRN. In the cytosol of the double mutant, exopolyphosphatase activity was practically absent under all of the above cultivation conditions. The content of polyphosphates in the cytosol preparations of all strains under study substantially decreased under P_i deficit. Transfer from P_i-deficient to P_i-rich medium was accompanied by polyphosphate over-accumulation only in the cytosol preparations of stains CRX and CNX, where their levels increased ~1.3 and 3.5-fold, respectively. No over-accumulation was observed in the parent strain CRY and in the *PPN1*-deficient strain CRN. These data suggest that the exopolyphosphatases encoded by the *PPX1* and *PPN1* genes are not involved in polyphosphate synthesis.

Key words: polyphosphates, exopolyphosphatases, cytosol, *PPX1* and *PPN1* mutants, *Saccharomyces cerevisiae.*

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Inorganic polyphosphates (polyP) consisting of phosphoric acid residues linked by high-energy phosphoanhydride bonds perform many functions in the cells of living organisms and are an important component of the system of phosphate homeostasis [1, 2]. In yeasts, the encoding genes are known for only two enzymes of polyP metabolism, exopolyphosphatases Ppx1 and Ppn1 [2–4]. The question about the role of these enzymes in phosphate homeostasis is still open.

The conditions of the so-called phosphate overcompensation, when the cells after phosphate starvation are transferred onto a medium enriched with this component, have an essential effect on polyP metabolism in yeasts. Under such conditions, they accumulate polyP in higher amounts than the control cells grown in a complete medium [1]. The phenomenon of overcompensation in yeasts has been well studied in whole cells [1]. There are also fragmentary data on the effect of overcompensation on the polyP content in vacuoles [5] and mitochondria [6] of the wild type strain of *S. cerevisiae*. The cytosol of yeast cells contains a substantial amount of polyP and, depending on cultivation conditions, also exopolyphosphatases Ppx1 and Ppn1; this is an indication of an important role of this compartment in the maintenance of P_i homeostasis in yeast cells.

The goal of the present work was to study the effect of inactivation of the *PPX1* and *PPN1* genes on the polyP content and exopolyphosphatase activities in the cytosol of *S. cerevisiae* at an abrupt change of P_i concentration in the cultivation medium.

MATERIALS AND METHODS

Saccharomyces cerevisiae strains CRY (the parent strain), CRX (the strain with inactivated *PPX1* gene), CRN (the strain with inactivated *PPN1* gene), and CNX (the strain with inactivated *PPX1* and *PPN1* genes) were obtained from the laboratory of Arthur Kornberg (Stanford University, United States) [4]. All strains were grown under aerobic conditions on a shaker at 30°C on YPD medium (1% yeast extract, 2% peptone, and 2% glucose) containing 5.7 mM orthophosphate (P_i-rich medium). The media (250 ml) were inoculated with yeast cultures collected directly from YPD agar

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Table 1. Exopolyphosphatase activities (mU/mg of protein) in the cytosol of *S. cerevisiae*

Yeast strains	Cultivation conditions		
	Stationary growth phase on P_i-rich medium	Stationary growth phase on P_i-deficient medium after transfer from P_i-rich medium	Active growth phase on P_i-rich medium after transfer from P_i-deficient medium
Parent strain CRY	130	190	325
Strain CRX with inactivated *PPX1* gene	80	80	135
Strain CRN with inactivated *PPN1* gene	30	90	140
Strain CNX with inactivated *PPX1* and *PPN1* genes	<5	<5	<5

Note: For the analysis of exopolyphosphatase activity, the cytosol was obtained without heparin and EDTA.

slants; the initial A_{600} was ~0.2. The optical density was measured on a SF-26 spectrophotometer in a 1-cm cuvette. Yeast cells were cultivated to the stationary growth phase for 24 h. The growth curves which are similar for all strains are presented in the work [7]. The cells were then precipitated at 5000 *g* for 10 min, washed with sterile distilled water, and transferred to 250 ml of P_i-deficient YPD medium; the initial A_{600} was ~0.2. The P_i-deficient medium containing 0.08 mM P_i was prepared according to the method described previously [8]. The growth curves for all strains on P_i-deficient and P_i-rich medium were identical.

For obtaining the cells in the phase of active growth, after 24-h cultivation (stationary growth phase) the cells were precipitated, washed with sterile distilled water, and inoculated into P_i-rich medium to the initial A_{600} of ~5 [7, 9]. After 2.5 h, when A_{600} of all cultures reached 10, the cells were precipitated, washed with distilled water, and used for further analysis.

Cytosol fractions were obtained by disruption of yeast spheroplasts in 0.1 M sorbitol followed by centrifugation at 100000 *g* for 3 h as described previously [10]. The cytosol preparations had no activity of ATPases sensitive to 100 μM orthovanadate (inhibitor of the ATPase of cytoplasmic membranes), 5 mM azide, 10 μg/ml oligomycin (inhibitors of the mitochondrial ATPase), and 50 mM nitrate (inhibitor of the vacuolar ATPase). Consequently, the cytosol preparations were not contaminated by respective organelles. The ATPase activity was detected by P_i release in 50 mM Tris–HCl, pH 7.2, containing 1 mM ATP and 2.5 mM $MgSO_4$.

The isolation of spheroplasts, as well as polyP extraction and detection have been described in our previous publications [9, 10]. With the purpose of obtaining cytosol preparations for polyP analysis, spheroplasts were destroyed in the presence of heparin (4 mg/ml) and EDTA (20 mM), known as exopolyphosphatase inhibitors [3]. Then an equal volume of 1 N $HClO_4$ was added to the fractions and the suspension was centrifuged at 5000 *g* for 20 min. The polyP from the spheroplasts of the above yeast are represented mainly by the acid-soluble fraction [6, 10]; only this fraction was therefore analyzed. For the analysis of exopolyphosphatase activity, cytosol fractions were obtained without heparin and EDTA. The exopolyphosphatase activity was determined by the rate of P_i production at 30°C during 20–30 min in 1 ml of the reaction mixture containing 50 mM of Tris–HCl, pH 7.2, 0.1 mM $CoSO_4$, and 9.6 μM $polyP_{208}$ (Monsanto, United States) as a polymer. The amount of the enzyme producing 1 μmole of P_i in 1 min was taken as a unit of activity (U). The mean values of three experiments are presented.

RESULTS AND DISCUSSION

Exopolyphosphatase activities depending on cultivation conditions were determined in the cytosol preparations of four different *S. cerevisiae* strains (Table 1). Under P_i deficit in the medium, a substantial increase of exopolyphosphatase activity was noted only for the strain CRN with an inactivated *PPN1* gene and for the parent strain CRY (3- and ~1.5-fold, respectively; Table 1). No change of exopolyphosphatase activity under P_i deficit was observed in the CRX strains with the inactivated *PPX1* gene and in the double mutant CNX. At transfer from a P_i-deficient to P_i-rich medium, the exopolyphosphatase activities in the cytosol preparations of CRY, CRX, and CRN increased ~1.7-fold (Table 1). However, such an increase of exopolyphosphatase activity was observed in the cytosol preparations of these strains at the transition to active growth on P_i-rich media [9, 11]. In other words, this increase of exopolyphosphatase activity is associated with the growth phase, rather than with an increase of P_i concentration in the cultivation medium.

The polyP content in the cytosol preparations of all *S. cerevisiae* strains under study decreased drastically under P_i deficit in the cultivation medium (Table 2). The cytosol of the parent strain CRY contains both exopolyphosphatases, Ppx1 and Ppn1; the CRX strain has Ppn1

Table 2. The content of polyphosphates (μmol P_i/mg of protein) in the cytosol of *S. cerevisiae* depending on cultivation conditions

Yeast strains	Cultivation conditions		
	Stationary growth phase on P_i-rich medium	Stationary growth phase on P_i-deficient medium after transfer from P_i-rich medium	Active growth phase on P_i-rich medium after transfer from P_i-deficient medium
CRY	0.8	0.06	0.8
CRX	1.5	0.07	2.0
CRN	0.9	0.04	0.7
CNX	1.7	0.05	6.0

Note: The cytosol was obtained in the presence of heparin (4 mg per 1 ml) and EDTA (20 mM), known exopolyphosphatase inhibitors.

Table 3. Polyphosphate content (μmol P_i/g of dry biomass) in the spheroplasts of *S. cerevisiae* depending on cultivation conditions

Yeast strain	Cultivation conditions		
	Stationary growth phase on P_i-rich medium	Stationary growth phase on P_i-deficient medium after transfer from P_i-rich medium	Active growth phase on P_i-rich medium after transfer from P_i-deficient medium
CRY	770	80	780
CRX	770	50	910
CRN	690	40	670
CNX	725	35	2500

and the CRN strain has Ppx1 [3]. The double mutant CNX has neither of these exopolyphosphatases, and any new exopolyphosphatase activity is not induced under P_i deficit (Table 1). However, in all the strains during the stationary growth phase in P_i-deficient medium the polyP level was equally low. It is presently unknown which enzymes are responsible for polyP hydrolysis in the double mutant. In the double mutant CNX, polyP is probably hydrolyzed by endopolyphosphatase, the enzyme that cleaves long polyP chains into shorter fragments. Short-chain polyP could then be hydrolyzed by pyrophosphatase [12], a high content of which is present in the cytosol preparation of strain CNX.

At the transfer from a P_i-deficient to P_i-rich medium, the effect of polyP over-accumulation was observed only in the cytosol preparations of strains CRX and CNX; their content was ~1.3- and 3.5-fold higher, respectively, than in the stationary phase culture on P_i-rich medium (Table 2). The effect of polyP over-accumulation was observed neither in the parent strain CRY nor in the *PPN1*-deficient strain CRN (Table 2). In these strains, the polyP level returned to the initial value and no over-accumulation occurred. Assuming that polyP overcompensation in these strains takes place not in the cytosol but in other compartments, e.g., in vacuoles, we determined polyP content in the spheroplasts of these strains (Table 3). However, the same tendency was observed in the spheroplasts as well: no overcompensation occurred in strains CRY and CRN, and the polyP content in strains CRX and CNX increased 1.2- and 3.5-fold, respectively (Table 3). These data do not agree with the results of other works, where polyP overcompensation was registered not only in the cells of the wild type *S. cerevisiae* strain [13] but also in the spheroplasts obtained from this strain [6]. The differences between the strains are probably of great significance for manifestation of polyP over-accumulation. It should be taken into account that the parent strain CRY is mutant by quite a number of genes (genotype: *MATa*, *ade2*, *his3*, *leu2*, *trp1*, *ura3*) [4]. Although these genes have no direct relation to phosphorus metabolism, it is

not improbable that disturbance of polyP over-accumulation is associated with the pleiotropic effect of the above mutations.

The results obtained in the study of P_i deficit conditions suggest that polyP is hydrolyzed by the Ppx1 exopolyphosphatase in the cytosol of strains CRY and CRN and by Ppn1 in strain CRX. In strain CNX, this hydrolysis is supposedly associated with successive functioning of endopolyphosphatase and pyrophosphatase.

The problem of polyP synthesis in yeasts has not yet been solved, in spite of the long history of its study. In bacteria, polyP is synthesized mainly by polyphosphate kinase [2]. Such activity was found recently in the yeast *Candida humicola* [14]. In *S. cerevisiae*, low polyphosphate kinase activity was revealed only in the vacuoles [1]. The possibility of the synthesis of some part of polyP due to the proton-motive force is favored indirectly by the data of inhibitory analysis [1]. Many enzymes of phosphorus metabolism, such as polyphosphate kinase [2], ATPase, and pyrophosphatase [15], are reversible and capable of both hydrolysis and synthesis of respective substrates. Due to the ambiguity of the question as to what enzymes synthesize polyP in yeasts, possible reversibility of exopolyphosphatases and their involvement in polyP synthesis was suggested [1]. However, the results of the present work on polyP over-accumulation in the double mutant CNX deficient in both exopolyphosphatases Ppx1 and Ppn1 demonstrate that these exopolyphosphatases are not involved in polyP synthesis.

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