Biochemistry (Moscow), Vol. 68, No. 7, 2003, pp. 740-746. Translated from Biokhimiya, Vol. 68, No. 7, 2003, pp. 902-909.

Effect of *PPX1* Inactivation on the Exopolyphosphatase Spectra in Cytosol and Mitochondria of the Yeast *Saccharomyces cerevisiae*

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Received May 27, 2002

Abstract—Inactivation of *PPX1* encoding exopolyphosphatase PPX1 in *Saccharomyces cerevisiae* results in a change in the exopolyphosphatase spectrum in the yeast cells. In the *PPX1*-deficient strain, elimination of an ~45 kD exopolyphosphatase is observed in the cytosol, and activity of an exopolyphosphatase with molecular mass of ~830 kD increases fivefold. The latter activity differs greatly in properties from the low-molecular-mass enzyme of the parent strain. In the soluble fraction of the mutant mitochondria, exopolyphosphatase of ~45 kD characteristic of the soluble mitochondrial fraction in the parent strain is eliminated, and exopolyphosphatase with a molecular mass of ~440 to ~830 kD is found. On *PPX1* inactivation, a membrane-bound form of mitochondrial exopolyphosphatase is unaffected in its activity level and properties. Therefore, the membrane-bound exopolyphosphatase of mitochondria and the high-molecular-mass enzyme of the cytosol of *S. cerevisiae* are not encoded by the *PPX1* gene, unlike the soluble low-molecular-mass exopolyphosphatase of mitochondria, which is probably a product of this gene with a posttranslational modification. In the *PPX1* mutant, exopolyphosphatase properties in the cell as a whole undergo modifications including the ability to hydrolyze polyphosphates (polyP) with different polymer degree.

Key words: cytosol, mitochondria, soluble fraction, membrane-bound fraction, exopolyphosphatase, *Saccharomyces cerevisiae*

The enzymes of inorganic polyphosphate metabolism play an essential role in the regulation of biochemical processes increasing cell tolerance of unfavorable environmental conditions and various stresses [1-4]. Availability of a great number of exopolyphosphatases (polyphosphate phosphohydrolase, EC 3.6.1.11) differing in their localization and physicochemical properties in the cells of *S. cerevisiae* [5-10] raises a question whether these forms are the products of one or several genes. To answer this question by the standard procedure of mutant production is problematic, since there is no possibility of their rapid screening. In the literature, however, a mutant of *S. cerevisiae* in the *PPX1* gene encoding one exopolyphosphatase form was described [11]. This mutant was obtained by sequencing and further disruption of the corresponding gene. Exopolyphosphatases from different compartments of the mutant cell were not analyzed.

The cytosol exopolyphosphatase contributes significantly to the total exopolyphosphatase activity of the yeast cell. Two exopolyphosphatase forms, membrane-bound and soluble, were detected in the mitochondria of *S. cerevisiae* [12]. There is special interest in polyphosphate metabolism in mitochondria because these polymers are important for stress response, and mitochondria, according to current concepts, seem to play a key role in this adaptation [13].

The present work demonstrates that inactivation of the *PPX1* gene results in a change in the exopolyphosphatase spectra of the cytosol and mitochondria.

MATERIALS AND METHODS

Yeast strains and cultivation conditions. Strains CRY (a parent strain) and CRX (a strain with inactivated *PPX1* gene) of the yeast *S. cerevisiae* were kindly provided by N. Rao from A. Kornberg's laboratory (Stanford University, USA). Both strains were grown in a medium with 1% yeast extract, 2% peptone, and 2% glucose as described earlier [11].

Isolation and sub-fractionation of mitochondria. Isolation of spheroplasts and mitochondria from the yeast

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S. cerevisiae (strains CRY and CRX) were described earlier [12]. Disintegration of spheroplasts for mitochondrial isolation was carried out in the presence of 0.05% concanavalin A from Pharmacia (Sweden). Isolated mitochondria were suspended in 20 mM Tris-HCl, pH 6.8, containing 1 mM EDTA, 4 mM $MgSO_4$, and 1 mM phenylmethylsulfonyl fluoride (PMSF).

Sub-fractionation of mitochondria for soluble and membrane fractions was carried out by sonication followed by membrane sedimentation as described earlier [12].

Cytosol preparation was obtained with disintegration of protoplasts by their osmotic lysis in the cold in 20 mM Tris-HCl, pH 7.2, containing 0.1 M sorbitol. To pellet the membrane fraction, the suspension was centrifuged for 60 min at 13,000*g* [6].

Exopolyphosphatase, tripolyphosphatase, and pyrophosphatase activities were determined by the rate of P_i formation at 30°C for 30-60 min in 1 ml of reaction mixture containing 50 mM Tris-HCl, pH 7.2, 2.5 mM $MgSO_4$, and 1 mM $polyP_{188}$, 1 mM $polyP_3$, or 1 mM pyrophosphate, respectively [7]. Phosphohydrolase activities in the mitochondrial preparation were measured with addition of 0.1% Triton X-100 in the incubation mixture. P_i formed during the reaction was determined with ascorbic acid and SDS [14]. For study of exopolyphosphatase and tripolyphosphatase activities, an activity unit (U) was defined as the enzyme quantity sufficient to catalyze the formation of 1 μmol P_i in 1 min. PolyP with the average chain length of 188 orthophosphate residues (Monsanto, USA) prepurified by gel filtration on Sephadex G-10 [5], tripolyphosphate, and pyrophosphate (Sigma, USA) were used in the work.

Protein concentrations were determined by a modified Lowry method [15] using bovine serum albumin as the standard.

Determination of exopolyphosphatase molecular masses. The cytosol preparation and the preparation of soluble mitochondrial fraction were loaded on a column (1.6 × 90 cm) with Sephacryl S-300 (Pharmacia), equilibrated with 20 mM Tris-HCl, pH 7.2, containing 0.1% Triton X-100, 0.1 M NaCl, 2 mM $MgSO_4$, and then eluted with the same buffer at 15 ml/h. Fraction volume was 3 ml. A molecular weight marker kit (Sigma) was used for calibration of the column.

RESULTS AND DISCUSSION

Cytosol exopolyphosphatases. Since the exopolyphosphatase product of the *PPX1* gene [11] was similar to that purified from the cytosol [16] in its properties, we first clarified the question how inactivation of the gene affected the cytosol exopolyphosphatase.

The growth patterns both of the parent strain CRY and the strain CRX with elimination of the *PPX1* gene encoding the ~45 kD exopolyphosphatase of *S. cerevisiae* were essentially alike in glucose-peptone medium. At the end of logarithmic growth phase, the total exopolyphosphatase activity in spheroplast lysate of the mutant CRX was only twice lower than in case of the CRY strain. The specific activity in the mutant lysate was half as much as in the CRY strain: 64 mU/mg protein, as compared with 140 mU/mg protein in the parent strain. The assumption that the main contribution to exopolyphosphatase activity of the *PPX1* mutant is due to the vacuolar enzyme [11] lacks support. It turned out that the cytosol of the *PPX1*-deficient mutant contained a large amount of exopolyphosphatase activity: 1.9 U/g as compared with 4.5 U/g wet biomass in the parent yeast strain. The contribution of exopolyphosphatase activity of the cytosol to the total cell activity was nearly the same for both strains: 52 and 46% for the parent strain and the mutant, respectively.

The studies of gel filtration of the cytosol preparations revealed that on inactivation of *PPX1* the enzyme PPX1 with the molecular mass of ~45 kD was really eliminated from the cytosol (Fig. 1). At the same time, the activity of a new enzyme of ~830 kD, which contributed no more than 10% to the total exopolyphosphatase activity in the cytosol of the parent strain, increased considerably in the mutant (Fig. 1).

The high-molecular-mass exopolyphosphatase turned out to differ considerably in its properties from the 45-kD enzyme of the cytosol. This enzyme was inhibited by 1 mM EDTA by 25-30%, while the 45-kD one was stimulated with this effector by 40% (Table 1). The high-molecular-mass enzyme was insensitive to antibodies

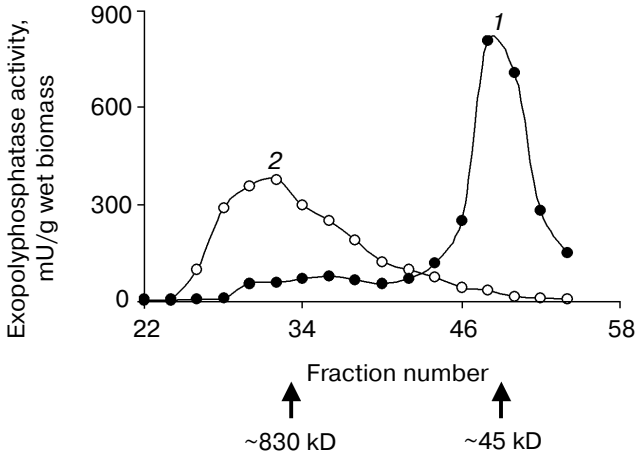

Fig. 1. Gel filtration of the cytosol preparations obtained from the parent strain (*1*) and *PPX1* mutant (*2*) of the yeast *S. cerevisiae* using Sephacryl S-300 column.

Table 1. Effect of some reagents on exopolyphosphatase activities in the cytosol of the yeast *S. cerevisiae* (CRY, parent strain; CRX, *PPX1* mutant; measurements were carried out in fractions obtained by gel filtration)

Reagent	Concentration	Activity, %		
		CRY		CRX
		~45 kD	~830 kD	~830 kD
No addition		100[a]	100[b]	100[c]
Heparin	20 μg/ml	5	10	10
NaF	1 mM	98	51	49
EDTA	1 mM	140	75	65
Antibodies	5 μl/ml	22	108	115

Note: Antibodies were obtained against purified 40-kD cell-envelope exopolyphosphatase of *S. cerevisiae* VKM Y-1173. The values of specific activities (mU per mg protein) corresponding to 100% were 230 (a), 55 (b), and 85 (c).

against purified cell-envelope exopolyphosphatase, while the low-molecular-mass enzyme was inhibited with them by ~80% (Table 1). Fluoride (1 mM), an inhibitor of pyrophosphatases, suppressed the high-molecular-mass enzyme by ~50% and was ineffective in the case of the low-molecular-mass enzyme of the parent strain (Table 1). Inhibition with fluoride seems to reflect the properties of the enzyme itself, since the preparations used for analysis had minor pyrophosphatase activities. Both enzyme activities were suppressed by heparin, a known inhibitor for the most types of exopolyphosphatases, by 90-95% (Table 1).

The substrate specificities of the two enzymes differed significantly from each other: the low-molecular-mass exopolyphosphatase was most active with tripolyphosphate, unlike that with the molecular mass of ~830 kD, which preferred the high-molecular-mass polyphosphates (Table 2). The major cytosolic exopolyphosphatase of the *PPX1*-deficient strain resembled in its properties the one replacing the low-molecular-mass enzyme in the cytosol of the yeast *S. cerevisiae* VKM Y-1173 after its transfer from P_i-limited cultivation medium to that containing P_i excess (conditions of polyphosphate overcompensation) [17].

Table 2. Substrate specificity of the cytosol exopolyphosphatases of the parent (CRY) and *PPX1*-deficient (CRX) strains of *S. cerevisiae* (measurements were carried out in the preparations obtained by gel filtration)

Substrate	Activity, mU per mg protein		
	CRY		CRX
	~45 kD	~830 kD	~830 kD
$PolyP_3$	250	60	70
$PolyP_{15}$	235	65	90
$PolyP_{208}$	165	70	100

Thus, the 45-kD exopolyphosphatase characteristic for the cytosol of the parent strain disappeared from the cytosol of *PPX1*-deficient mutant. Hence, the cytosol exopolyphosphatase purified earlier [16] is encoded by the *PPX1* gene. The content of exopolyphosphatase with molecular mass of ~830 kD increased in the mutant CRX as compared with the cytosol of the parent yeast strain. Consequently, this enzyme is not encoded by the *PPX1* gene. It seems likely that there is a certain coordinated mechanism of expression regulation of these two enzymes. Considering that the cytosol exopolyphosphatases contribute significantly to the total enzyme activity of a cell, it should be mentioned that in the mutant the ability to hydrolyze polyphosphates with different polymeric degrees undergoes modification in the cell as a whole, just as was observed on overcompensation at a stationary growth phase [17].

Mitochondrial exopolyphosphatases. The soluble exopolyphosphatase of mitochondria was similar to that encoded by the *PPX1* gene in molecular mass and substrate specificity. To clear up the question as to whether it was encoded by the *PPX1* gene, some properties of soluble and membrane-bound exopolyphosphatase activities

Table 3. Effect of various inhibitors on ATPase activities of mitochondrial preparations from the CRY and CRX strains of the yeast *S. cerevisiae* (the average value of four independent experiments is given)

S. cerevisiae strain	ATPase activity, U per mg protein				
	pH 8.5		pH 7.2		
	control	sodium aside (5 mM)	control	sodium nitrate (50 mM)	sodium orthovanadate (100 mM)
CRX	1.6	0.15	0.96	1.1	0.96
CRY	1.5	0.22	1.0	1.2	1.05

Table 4. Exopolyphosphatase and pyrophosphatase activities of soluble and membrane fractions of mitochondria isolated from CRY and CRX strains of the yeast *S. cerevisiae* (the average value of four independent experiments is given)

Enzyme activity	Activity, % of total activity of mitochondrial fraction			
	CRY		CRX	
	soluble fraction	membrane fraction	soluble fraction	membrane fraction
Pyrophosphatase	67	33	79	21
Exopolyphosphatase	23	77	21	79

of mitochondria were compared in the strains CRY and CRX. Inhibitor analysis of mitochondrial ATPase showed that the preparations of mitochondria obtained from both yeast strains were reasonably free from contamination by plasma membrane and vacuoles (Table 3). ATPase activity of mitochondrial preparations was effectively suppressed with sodium aside, an inhibitor of the F-type ATPases [18]. Yet, it was insensitive to the action of orthovanadate, a specific inhibitor of the yeast plasma-membrane H^+-ATPases, and nitrate, an inhibitor of the vacuolar H^+-ATPases [19, 20]. Under sub-fractionation of isolated mitochondria for soluble and membrane fractions, the distribution of pyrophosphatase activity between these preparations in both strains was similar to that obtained earlier for the yeast *S. cerevisiae* VKM Y-1173 [12]. In both strains, most of this activity was found in the soluble fraction. A major portion of the exopolyphosphatase activity of mitochondria isolated from cells in the late logarithmic growth phase was found in the membrane fraction of both strains (Table 4).

In both strains, the biochemical properties and specific activities were equal (Table 5) and similar to those found for the membrane exopolyphosphatase of *S. cerevisiae* VKM Y-1173 [12], that is, the inactivation of the *PPX1* gene had no effect on the membrane mitochondrial exopolyphosphatase. Membrane-bound mitochondrial enzymes of both strains conserved a unique ability to be inhibited by Mg^{2+}, which was found for *S. cerevisiae* VKM Y-1173, unlike the soluble mitochondrial exopolyphosphatase, which was stimulated by bivalent metal cations (not illustrated).

Some properties of exopolyphosphatase activities of the soluble mitochondrial fractions of both the parent and mutant strains were similar: they were sensitive to the action of heparin and were not suppressed by antibodies that inhibited the 45-kD exopolyphosphatase of the cytosol (Table 5). However, some distinctions were observed. First, exopolyphosphatase of the soluble fraction of the mutant was inhibited by fluoride (Table 5). Only the vacuolar and high-molecular-mass exopolyphosphatases of the mutant cytosol (Table 1) were sensitive to fluoride. However, the mitochondrial preparation was devoid of vacuolar contamination (Table 3). Second, specific exopolyphosphatase activity of the soluble mitochondrial fraction of the mutant strain was variable. In some experiments, when cells were used at the earlier growth phase, it was considerably higher compared with that demonstrated in Table 4 and reached 146 mU per mg protein. This cast some doubt that the soluble mitochondrial fraction of the mutant contains the same enzyme as the parent strain.

To clear up this question, gel filtration of soluble mitochondrial preparations from the CRY and CRX strains was carried out using a Sephacryl S-300 column.

Table 5. Effect of some reagents on exopolyphosphatase activities of mitochondria isolated from parent strain CRY and *PPX1*-deficient CRX of *S. cerevisiae*

Reagent	Concentration	Activity, %			
		CRY		CRX	
		soluble fraction	membrane fraction	soluble fraction	membrane fraction
No addition		100[a]	100[b]	100[c]	100[d]
Heparin	20 μg/ml	0	0	0	0
NaF	1 mM	100	60	55	65
Antibodies	5 μl/ml	85	100	110	110
EDTA	1 mM	100	95	100	100

Note: Antibodies were raised against purified 40-kD cell envelope exopolyphosphatase of *S. cerevisiae* VKM Y-1173. The values of specific activities (mU per mg protein) corresponding to 100% were 65 (a), 35 (b), 85 (c), and 35 (d).

Under gel filtration of the soluble preparation of mitochondria isolated from the CRY strain, exopolyphosphatase activity corresponded to a protein of ~45 kD (Fig. 2), which correlates with the data obtained earlier [12]. The peaks of pyrophosphatase and tripolyphosphatase activities were similar to those found previously [12]. When the soluble preparation of mitochondria from the CRX strain was used, it turned out that the preparation lacked the exopolyphosphatase of ~45 kD and the enzyme activity was in the range of ~440-830 kD (Fig. 2). It is significant that in the case of the CRX strain, the peaks of exopolyphosphatase and tripolyphosphatase activities were not coincident, i.e., exopolyphosphatase substrate specificity was similar to that of the cytosol preparation of this strain. The gel filtration character was unchanged irrespective of whether the initial exopolyphosphatase activity in the soluble preparation of mitochondria was high or low.

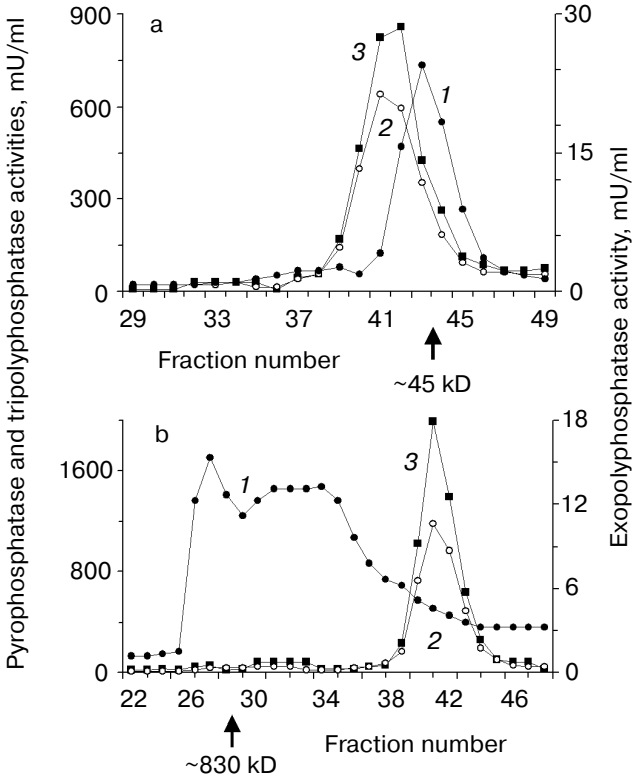

Fig. 2. Gel filtration of soluble preparation of mitochondria of the yeast *S. cerevisiae* grown to the middle of logarithmic growth phase using a column with Sephacryl S-300: a) CRY strain; b) CRX strain. Activities: exopolyphosphatase (*1*), pyrophosphatase (*2*), tripolyphosphatase (*3*).

The results of these experiments demonstrate that exopolyphosphatase activity of the soluble preparation of mitochondria isolated from the mutant is not associated with the 45-kD enzyme, as with the parent strain, but is associated with the high-molecular-mass exopolyphosphatase found in the cytosol of this mutant. The data suggest that the soluble exopolyphosphatase of the yeast mitochondria of ~45 kD is under the control of the *PPX1* gene encoding the major exopolyphosphatase of the cytosol. Despite the common genetic determinant, the mitochondrial and cytosolic forms of the low-molecular-mass enzyme vary in a number of properties. The soluble low-molecular-mass exopolyphosphatase of mitochondria from the parent strain is not inhibited by antibodies against the purified cell envelope exopolyphosphatase, which suppressed the cytosol enzyme by 80%. It is not stimulated by EDTA, in contrast to the low-molecular-mass enzyme of the cytosol (Table 1), and differs from it in kinetic properties [4, 12]. Consequently, the presence of the soluble low-molecular-mass exopolyphosphatase in the yeast mitochondria isolated from the CRY and

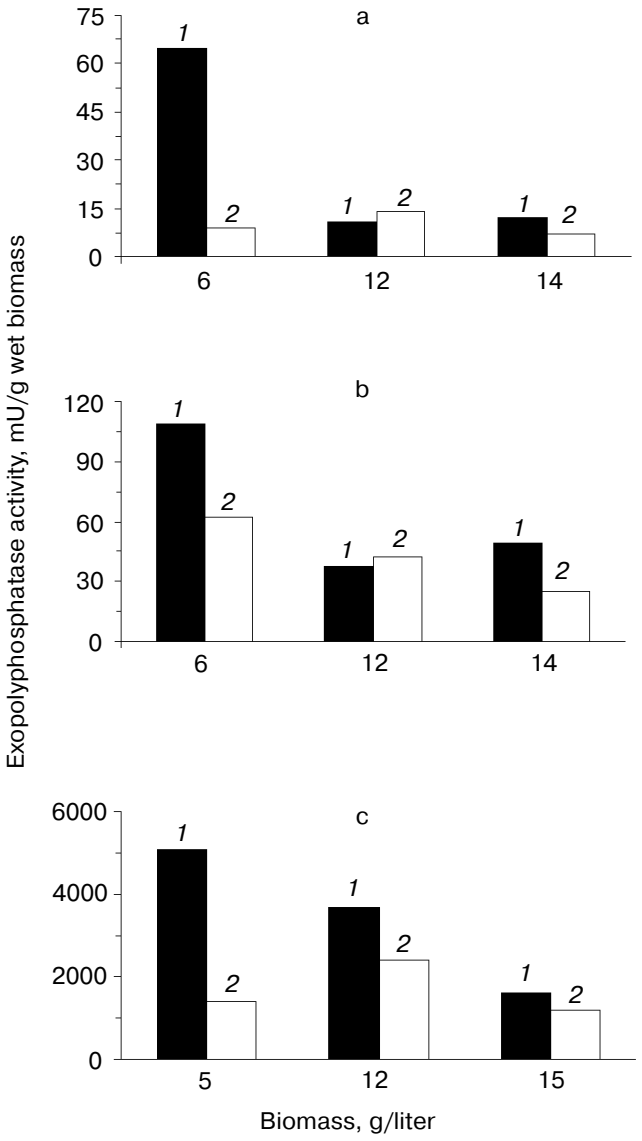

Fig. 3. Exopolyphosphatase activities of soluble (a) and membrane (b) fractions of mitochondria and in the cytosol (c) at early, middle, and late logarithmic growth phases: *1*) CRY strain; *2*) CRX strain.

VKM Y-1173 strains is not due to contamination of the preparation with the cytosolic form of the enzyme. The above distinctions could be explained by post-translational modification of the enzyme. This modification and possible block of N-terminal sequence of the protein might account for no evidence of the *PPX1* sequence in the preparation of purified enzyme [21]. The wrong addressing of some proteins to mitochondria is well known: no less than 10% of accidental signal sequences may serve as mitochondrial addressing signals [22]. Probably, the presence of exopolyphosphatase, characteristic of the cytosol of the mutant, in mitochondrial preparation of the same strain is also caused by the above phenomenon. It should be mentioned that mitochondria contain no more than 5% of the total exopolyphosphatase activity of the cell in both the parent and mutant strains.

Effect of growth phase on exopolyphosphatase activities of the cytosol and mitochondria in CRY and CRX strains. In both strains, exopolyphosphatase activities of the cytosol and both soluble and membrane preparations of mitochondria were compared in cells at different growth phases (Fig. 3). Exopolyphosphatase activity of the cytosol from the CRY strain decreased during growth, while that in the CRX strain was little affected, with a minor maximum in the middle of logarithmic phase. The dependence of low-molecular-mass and high-molecular-mass exopolyphosphatases of the soluble mitochondrial preparation on the growth phase was similar to those in the cytosol in both the parent and mutant strains. This correlates well with the data that the level of enzyme activity found in the soluble mitochondrial preparations of both strains was associated with their level in the cytosol. Activity of the membrane-bound exopolyphosphatase of mitochondria decreased during the growth in both strains.

Thus, the experiments indicate that 45-kD exopolyphosphatases of both the cytosol and soluble mitochondrial preparations are encoded by the *PPX1* gene. The distinction between the properties of the soluble exopolyphosphatase of mitochondria and the high-molecular-mass exopolyphosphatase found in the cytosol might be due to modification of this protein. Both the membrane-bound exopolyphosphatase of mitochondria and high-molecular-mass enzyme found in the cytosol and mitochondria on inactivation of the above gene have their own determinants. It should be mentioned that there is a change in exopolyphosphatase properties of cell as a whole in the *PPX1*-deficient mutant, including the ability to hydrolyze polyphosphates with different chain length.

This work was supported by grants from the Russian Foundation for Basic Research (Nos. 02-04-48544 and 01-04-97021).

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