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Effect of Inhibitors on Polyphosphate Metabolism in the Yeast *Saccharomyces cerevisiae* under Hypercompensation Conditions

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Abstract—After re-inoculation of the yeast *Saccharomyces cerevisiae* from phosphate-deficient to complete medium, the total content of polyphosphates increased tenfold during 2 h (hypercompensation), but the content of certain fractions increased differently. The content of acid-soluble polyphosphate increased to the maximal extent. The ratio of the activities of two exopolyphosphatases also changed in the cytosol. Activity of a low molecular weight exopolyphosphatase (40 kD) decreased almost twice, whereas activity of a high molecular weight exopolyphosphatase (830 kD) increased tenfold. Cycloheximide blocks the increase in activity of high molecular weight exopolyphosphatase and hence, under these conditions the latter is synthesized *de novo*. Inhibitors of energy metabolism and cycloheximide, an inhibitor of protein synthesis, differently influence accumulation of certain polyphosphate fractions under hypercompensation conditions. The effect of iodoacetamide, an inhibitor of glycolysis, on any fraction is negligible, while cycloheximide suppresses accumulation of only polyP4 fraction associated with the cell envelope and bafilomycin A1, an inhibitor of vacuolar H^+-ATPase, suppresses accumulation of polyP3 fraction. The protonophore carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) to variable extent inhibits accumulation of all the fractions. Analysis of the effect of inhibitors on accumulation of polyphosphates under hypercompensation conditions confirms various localization, heterogeneity, and multiplicity of the routes of biosynthesis of certain fractions of these macroergic phosphorus compounds and also suggests interrelation between their biosynthesis and the gradient of H^+ electrochemical potential.

Key words: polyphosphates, exopolyphosphatase, cytosol, localization, hypercompensation, yeast, *Saccharomyces cerevisiae*, metabolism inhibitors

Metabolism of inorganic polyphosphates (polyP) having multiple energetic and regulatory functions in procaryotic and eucaryotic cells changes significantly depending on environmental conditions [1-6]. In yeast the polyP content drastically decreases under phosphate-deficient conditions and several times increases under phosphate-hypercompensation conditions, when the cells are transferred from phosphate-deficient to complete medium [1, 7]. Mechanisms providing regulation of polyP concentration in yeast cells and the role of exopolyphosphatases (polyphosphate phosphohydrolases, EC 3.6.1.11) are still unstudied. Under hypercompensation conditions, the polyP content in the cytosol changes significantly [4]. On re-inoculation from phosphate-deficient to complete medium, the variety of exopolyphosphatases in this compartment also changes: in addition to the 40-kD exopolyphosphatase most actively hydrolyzing tripolyphosphate and nucleoside tetraphosphates, a novel high molecular weight exopolyphosphatase specific to high molecular weight polyP appears [5, 8, 9].

The goal of this work was to analyze the effect of various inhibitors on polyP accumulation under hypercompensation conditions and to clarify possible participation of high molecular weight exopolyphosphatase of the cytosol in accumulation of polyP.

MATERIALS AND METHODS

Saccharomyces cerevisiae yeast BKM Y-1173 was grown on a shaker in flasks with 200 ml of Reader medium at 30°C [10]. Complete Reader medium with 18.3 mM P_i (+P) as well as phosphate-deficient Reader medium with only 1.3 mM P_i (−P) were used [8]. This

Abbreviations: polyP) inorganic polyphosphates; FCCP) carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; $\Delta\mu_{H^+}$) gradient of H^+ electrochemical potential.

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small amount of phosphate was caused by its concentration in the commercial yeast extract preparation (Serva, Germany). The yeast biomass grown to the late logarithmic phase (A_{600} = 5) on Reader medium (–P) was collected by centrifugation at 5000*g* for 10 min, washed with distilled water, and placed on fresh complete medium (+P) so that the initial density of the culture remained the same (A_{600} = 5). Inhibitors were added to the culture medium at the time of re-inoculation. After cultivation for 2 h, the cells were centrifuged, washed with distilled water, and used for further analysis.

Cytosol preparations [11] and various polyP fractions [7] were obtained as described earlier. The following polyP fractions were obtained: acid-soluble (polyP1), salt-soluble (polyP2), and two alkali-soluble (polyP3 and polyP4). The polyP5 fraction was determined via formation of P_i on hydrolysis of biomass remaining after preceding extractions by 1 M $HClO_4$ for 20 min at 90°C. Exopolyphosphatase activity was estimated via the rate of formation of P_i as described earlier [12]. The reaction mixture contained 50 mM Tris-HCl buffer, pH 7.2, 2.5 mM $MgSO_4$, and 0.005 mM $polyP_{208}$. One activity unit (U) was defined as the enzyme quantity sufficient to catalyze the formation of 1 μmol P_i in 1 min. $PolyP_{208}$ (Monsanto, USA) was preliminarily purified from P_i and PP_i by gel filtration on Sephadex G-10 [13].

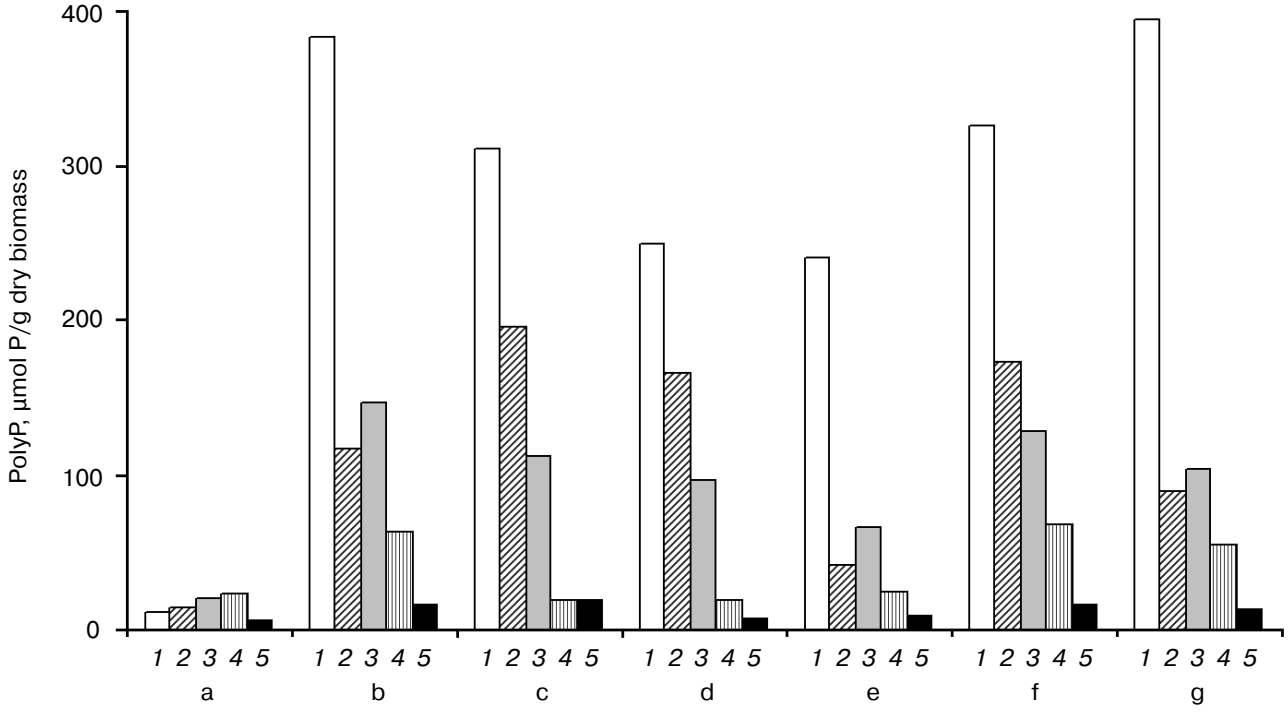
Exopolyphosphatases of the cytosol were separated by gel filtration on a 1.6 × 80 cm Sephacryl S-300 column (Pharmacia, Sweden) equilibrated with 20 mM Tris-HCl buffer, pH 7.2, containing 2 mM $MgSO_4$, 100 mM NaCl, and 0.1% Triton X-100 [8]. To estimate the total activity of each exopolyphosphatase, the pooled fractions of the corresponding peaks were used. Ribonuclease (13.7 kD), chymotrypsinogen A (25 kD), ovalbumin (43 kD), bovine serum albumin (67 kD), catalase (232 kD), ferritin (440 kD), and thyroglobulin (660 kD) (Sigma, USA) were used as standards.

RESULTS AND DISCUSSION

After re-inoculation of *S. cerevisiae* yeast cells from (–P) medium to (+P) medium, hypercompensation was observed already after 2 h: the total polyP content increased ~10 times, whereas the P_i content increased less than twofold (table). The polyP1, polyP2, and polyP3 fractions increased to the most. The polyP4 and polyP5 fractions increased 2-3 times (figure, panels (a) and (b)). As shown by gel filtration, after cultivation for 2 h a high molecular weight exopolyphosphatase was found in the cytosol; earlier it was observed under hypercompensation conditions at the later growth phases [8]. Two fractions with exopolyphosphatase activity were obtained from the cytosol preparation. One of them contained exopolyphosphatase I, the earlier studied enzyme with molecular mass 40 kD [9]. The second enzyme,

Effect of inhibitors on the total activity of exopolyphosphatases I and II in *S. cerevisiae* cytosol and also on P_i and polyP content in whole cells under hypercompensation conditions (cells grown on phosphate-deficient medium (–P) to late logarithmic phase were re-inoculated to complete medium (+P) containing various effectors)

Growth time after re-inoculation, h	Effectors	Dry biomass, g/liter	Total activity, U/g dry biomass		P_i, μmol P/g dry biomass	PolyP, μmol P/g dry biomass
			exopolyphosphatase I	exopolyphosphatase II		
0	without additions	1.5	18.5	1.1	100	75
2	without additions (control)	2.9	10.3	10.2	170	730
	cycloheximide (10 μg/ml)	1.6	14.5	3.4	180	660
	FCCP (10 μM)	2.1	13.0	14.0	120	540
	FCCP (20 μM)	2.0	10.5	2.9	80	380
	iodoacetamide (250 μM)	1.9	10.0	20.5	160	710
	bafilomycin A1 (0.05 μM)	2.7	11.2	12.8	180	660



Effect of inhibitors on the content of certain polyP fractions in *S. cerevisiae* cells under hypercompensation conditions. PolyP fractions: *1*) acid-soluble (polyP1); *2*) salt-soluble (polyP2); *3*, *4*) two alkali-soluble (polyP3 and polyP4); *5*) polyP5, determined via production of orthophosphate on hydrolysis of biomass remaining after extraction of preceding fractions with 1 M $HClO_4$ for 20 min at 90°C. a) Moment of re-inoculation; b-g) 2-h cultivation after re-inoculation from phosphate-deficient to complete medium in the absence of inhibitors (control) (b) or in the presence of cycloheximide (10 μg/ml) (c), 10 μM FCCP (d), 20 μM FCCP (e), 250 μM iodoacetamide (f), 50 nM bafilomycin A1 (g)

exopolyphosphatase II, has molecular mass 830 kD (data not presented here). As shown earlier, these enzymes differ in substrate specificity and sensitivity to bivalent metal cations, EDTA, fluoride, and antibodies against the purified cell envelope exopolyphosphatase [8]. During 2 h cultivation the contribution of exopolyphosphatase II to the total exopolyphosphatase activity in the cytosol increased from 5.6 to 50% (table), and after cultivation for 16 h to 90% [8].

To study the effect of inhibitors on the hypercompensation process, after re-inoculation the yeast was cultivated for 2 h in the presence of inhibitors, because this time is sufficient for accumulation of polyP and change in the spectrum of cytosol exopolyphosphatases.

Cycloheximide, an inhibitor of protein synthesis, at the used concentration suppressed the yeast growth almost completely (table); however, increase in the total polyP and P_i contents were almost equal to those in control (table). Only a certain redistribution between the fractions occurred: polyP1 and polyP3 content appeared to be somewhat lower, and polyP2 content higher than in control (figure, panels (b) and (c)). Only accumulation of polyP4 fraction appeared to be disrupted; in the presence of cycloheximide its content did not increase compared with the time of re-inoculation and appeared to be markedly lower than in control (figure). Accumulation of polyP4 fraction which does not contribute significantly to the total polyP accumulation at hypercompensation is considered to be related with the synthesis of mannoproteins of the cell envelope during cell growth blocked under these conditions [14, 15].

In the presence of cycloheximide the activity of exopolyphosphatase II increased to a significantly smaller extent compared with control (table). Consequently, increase in the activity of this enzyme is caused by its synthesis *de novo*. As shown above, disruption in the synthesis of exopolyphosphatase II in the presence of cycloheximide has negligible effect on accumulation of polyP in the cell. The data indicate that exopolyphosphatase II does not influence significantly the polyP synthesis at hypercompensation and enzymes catalyzing the synthesis of polyP1, 2, 3, and 5 were induced before re-inoculation. Depletion of cell polyP, during growth on (–P) medium may be a signal to their induction.

It is known that addition of the protonophore FCCP to the cultural medium results in efficient decrease in H^+

electrochemical potential ($\Delta\mu_{H^+}$) in plasmatic, vacuolar, and mitochondrial yeast cell membranes [16]. Under our experimental conditions FCCP suppressed cell growth already at concentration 10 μM (table). However, P_i content in the cells did not increase compared with that at the time of re-inoculation. The total polyP content appeared to be 26% lower than in control (table). At FCCP concentration 20 μM the total polyP accumulation decreased by 48% compared with control (table). Consequently, under hypercompensation conditions accumulation of at least some part of polyP is related with $\Delta\mu_{H^+}$. The polyP4 fraction appeared to be the most sensitive to FCCP. Its level did not increase already at 10 μM FCCP compared with the starting value on (−P) medium (figure, panels (a) and (d)); the data demonstrate the maximal interrelation with $\Delta\mu_{H^+}$ compared with other fractions. Dependence of accumulation of some polyP fractions on a gradient of ion concentration on *S. carlsbergensis* plasmatic membrane was also observed during cell adsorption of Mn^{2+} and Zn^{2+} [17]. The polyP1 fraction appeared to be the next in sensitivity to FCCP; its contribution to the total increase in polyP content at hypercompensation is maximal. Independent of FCCP concentration used, addition of the polyP1 content decreased by 40% compared with control (figure, panels (b), (d), (e)). This fraction of acid-soluble low molecular weight polyP is known to be heterogeneous in composition and localization [1, 3, 7]. We suppose that polyP comprising it is synthesized via various pathways, both related and not related with $\Delta\mu_{H^+}$. Only at twice larger FCCP concentrations, addition of the polyP2 and polyP3 content decreased significantly (figure, panels (b) and (e)). These fractions are considered to be localized in the surface-distant cell organelles including vacuoles and nuclei which are less accessible for protonophore [1, 3].

Unlike its inhibitory effect on the polyP accumulation, 10 μM FCCP caused some increase in the total exopolyphosphatase activity in cytosol compared with control. However, the ratio of two exopolyphosphatases changed almost similarly to that in control compared with the time of re-inoculation (table). Thus, this FCCP concentration did not disrupt the synthesis of exopolyphosphatase II, and its activity was even somewhat higher than in control. Only at 20 μM FCCP synthesis of this enzyme was blocked, maybe due to more complete de-energization of cell membranes which can cause disruption of signal transduction [18].

Iodoacetamide, a well-known inhibitor of glycolysis, first of all influencing triosephosphate dehydrogenase, efficiently suppresses yeast growth (table); however, its effect on the content of certain polyP fractions and their total quantity was negligible (table and figure, panels (b) and (f)). The total exopolyphosphatase activity of cytosol increased by ~50% at the cost of exopolyphosphatase II in the presence of both iodoacetamide and 10 μM FCCP.

Some authors consider that most of yeast cell polyP is localized in vacuoles [19, 20]. It was shown that under the normal growth conditions ^{31}P-NMR signal of polyP of whole yeast cell disappeared in the presence of bafilomycin A1, an inhibitor of H^+-ATPase of vacuolar membranes [16]. That is why we studied the effect of this inhibitor on the content of various polyP fractions under hypercompensation conditions. At the used concentration, bafilomycin A1 did not influence yeast growth (table). In the presence of this inhibitor, increase in the content of polyP1, polyP4, and polyP5 was the same as in control (figure, panels (b) and (g)), whereas the content of polyP2 was somewhat lower than in control. The effect of bafilomycin A1 was maximal in the case of polyP3 fraction; its content decreased by ~30% (figure, panels (b) and (g)). We supposed that synthesis of part of the polyP3 fraction depends on $\Delta\mu_{H^+}$ on vacuolar membrane. In the presence of this inhibitor, the activities and the ratio of exopolyphosphatases I and II were close to those in control (table).

Data on the effect of inhibitors on polyP accumulation in yeast cells are few and are mainly related with the total content and the content of certain fractions. It was shown that polyP accumulation in *S. cerevisiae* is suppressed by antimycin A if ethanol is used as an energy source and is not suppressed in the presence of glucose [21]. PolyP in yeast cells under various conditions are often studied by ^{31}P-NMR [3, 16, 22]. However, most of the yeast cell polyP cannot be detected by this method because of localization and pH of compartment and complex formation with cations and other cell components [4]. That is why conclusion about disappearance of all yeast cell polyP on de-energization of vacuolar membrane in the presence of protonophores and bafilomycin, based only on ^{31}P-NMR data, seems to be proved insufficiently [16]. A hypothesis about localization of most of the yeast cell polyP in vacuoles widespread in literature is only true for certain cultivation conditions [16, 19, 20]. In most cases polyP can be localized in almost all yeast cell compartments [1-6]. Thus, up to 70% of all cell polyP pool can be contained in yeast cytosol depending on cultivation conditions [4, 23].

In this work we analyzed the effect of inhibitors of energy metabolism (FCCP, iodoacetamide, bafilomycin A1) and cycloheximide, an inhibitor of protein synthesis, on accumulation of various polyP fractions under hypercompensation conditions. It should be noted that the used method of polyP extraction gives a more complete pattern of their content in yeast cells than ^{31}P-NMR and other known extraction methods [4]. Inhibitors are shown to unequally effect accumulation of certain polyP fractions. Thus, polyP4 appeared to be the only fraction whose accumulation was suppressed completely by both FCCP and cycloheximide. This confirms an idea that polyP4 has a specific pathway of biosynthesis and specific function in the cell [3, 14, 15]. Negligible effect of

cycloheximide on accumulation of other polyP fractions indicates that corresponding enzymatic systems were already induced during phosphorus deficit. The effect of FCCP demonstrated that energization of cell compartment membranes is important for accumulation of various polyP fractions. However, de-energization of vacuolar membrane by bafilomycin A1 mainly results in decrease in accumulation of only polyP3 fraction. So, the effect of inhibitors on polyP accumulation under hypercompensation conditions confirms that certain polyP fractions obtained by chemical extraction have different localization, heterogeneity, and variable pathways of biosynthesis.

The use of inhibitors distinguished two processes occurring after re-inoculation of the yeast from phosphate-deficient to complete medium: accumulation of polyP and synthesis *de novo* of high molecular weight exopolyphosphatase of cytosol. Thus, cycloheximide blocking the synthesis of this enzyme negligibly influenced polyP accumulation. In contrast, FCCP at concentration 10 μM decreasing polyP accumulation, even increased activity of high molecular weight exopolyphosphatase. So, high molecular weight exopolyphosphatase of cytosol does not seem to participate in the synthesis of polyP under hypercompensation conditions. Maybe this enzyme is necessary for the use of polyP accumulated in hypercompensation, for further yeast growth under the changed conditions. As for the low molecular weight exopolyphosphatase of this compartment, its activity decreased under hypercompensation conditions, and this decrease was negligibly affected by inhibitors. The role of this enzyme in the cell remains unclear. Vitality of mutants in the gene encoding this enzyme is not disrupted, and only combination of such mutation with mutation in the gene encoding endopolyphosphatase decreases survival of yeast cells in the stationary growth phase [24, 25].

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