ISSN 0026-2617, Microbiology, 2011, Vol. 80, No. 1, pp. 15–20. © Pleiades Publishing, Ltd., 2011.
Original Russian Text © V.M. Vagabov, L.V. Trilisenko, O.Yu. Kochetkova, A.P. Ilchenko, I.S. Kulaev, 2011, published in Mikrobiologiya, 2011, Vol. 80, No. 1, pp. 18 – 23.

EXPERIMENTAL ARTICLES

Effect of *m*-Carbonyl Cyanide 3-Chlorophenylhydrazone on Inorganic Polyphosphates Synthesis in *Saccharomyces cerevisiae* under Different Growth Conditions

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Received April 8, 2010

Abstract—The effects of different concentrations of the protonophore uncoupler *m*-carbonyl cyanide 3-hchlorophenylhydrazone (CCCP) on the synthesis of inorganic polyphosphates (polyP) during the first 0.5 h of hypercompensation in the yeast *Saccharomyces cerevisiae* VKM Y-1173 growing on media with 2% glucose under low (hypoxia) or high aeration or with 1% (vol/vol) ethanol under high aeration were studied. It was shown that the yeast growth on ethanol was completely inhibited by 5 μM CCCP, while growth on glucose was inhibited by 25 μM CCCP, independently of aeration of the medium. The maximum rate of H_2 absorption was shown at 2, 5, and 25 μM CCCP for the cells grown on ethanol, on glucose under high aeration, and on glucose under hypoxia, respectively. Against the decrease of total ATP level and total polyP, CCCP had a nonuniform effect on the synthesis of individual polyP fractions. CCCP maximally inhibited synthesis of the most actively formed fractions: polyPI during growth on glucose under hypoxia, polyPIII during growth on glucose under aeration, and polyPIII and polyPV during growth on ethanol. CCCP had no substantial effect on the synthesis of polyPII and polyPIV fractions, the formation of which seems to be less related to the electrochemical potential gradient of H^+ ions.

Keywords: inorganic polyphosphates, carbon source, aeration, CCCP, yeast, *Saccharomyces cerevisiae*.

DOI: 10.1134/S0026261711010176

Inorganic polyphosphates (polyP) are linear polymers consisting of orthophosphoric acid residues connected by an energy-rich phosphoanhydride bond. They perform numerous functions in the cell, including phosphate and energy reservation, formation of membrane channels in complex with poly–β-hydroxybutyrate, regulation of gene expression, maintenance of the necessary level of cations, and cell wall structure formation [1–4].

In yeasts and other eukaryotic organisms, polyP have been found in all of the major cell compartments (cytosol, nucleus, mitochondria, vacuoles, and cell wall), and their metabolism is closely related to the main processes occurring in these structures [1, 3, 4].

While the pathways of polyP degradation and the enzymes involved in these processes have been rather well studied in *S. cerevisiae* [3–5], many issues related to polyP synthesis are still open. *S. cerevisiae*, in contrast to *Candida humicola* [6], was not shown to contain a polyphosphate kinase (EC 2.7.4.1) performing polyP synthesis in bacteria using the terminal phosphate of ATP [2]. Recently it has been shown that the cytoplasmic domain of at least one of the vacuolar transport chaperons (Vtc4p) contains a 35-kDa segment possessing polyP polymerase activity. PolyP is synthesized from the terminal phosphate of ATP with formation of ADP. As opposed to polyphosphate kinase, this enzyme does not catalyze the reverse reaction of ATP synthesis from polyP [7]. It is still unclear whether the vacuolar polyP is formed in such a way.

The polyP localized in the yeast cell wall is synthesized with participation of dolichyl diphosphate-polyP phosphotransferase (EC 2.7.4.20) [8].

Since the polyP content of the cell wall and vacuoles together does not exceed 30–35% of their total content in the cell, it is clear that the way of formation of the main polyP pool in *S. cerevisiae* is still open.

PolyP content depends on yeast growth stage, carbon nutrient sources, and degree of aeration of the medium [9, 10]. It has been shown that inhibition of glycolysis by iodine acetamide and of oxidative phosphorylation by antimycin A at the level of the respiratory chain leads to a decrease in polyP synthesis [11, 12].

Evidence for a certain connection between polyP accumulation and electrochemical potential gradient of H^+ ions has been obtained [11, 13]. The level of vacuolar polyP detected by P^{31}-NMR spectroscopy decreased as a result of the effect of a protonophore

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uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP) on intact *S. cerevisiae* cells grown in a medium with glucose or lactate under anaerobic or aerobic conditions [13].

The vacuoles of *S. cerevisiae* contain low-molecular polyP with the chain length $n = 18–20$ orthophosphoric acid residues [14]. In yeast cells, a polyP chain may consist of 200–250 orthophosphoric acid residues [15].

Five polyP fractions with increasing polymer chain length can be isolated from yeast biomass. The synthesis of polyP of some fractions was shown to be associated preferably with different energy transduction processes. For example, it has been established that the most polymeric polyP of fraction V is actively synthesized by a culture growing on ethanol under high aeration of the medium. Under conditions favorable for glycolysis, mainly the polyP of fraction I is accumulated [9]. PolyP synthesis can be judged most correctly under conditions of short-term (no more than 30–40 min) hypercompensation (oversynthesis) of these compounds after transfer of the yeast cells from phosphate-free to P_i-enriched medium. The longer exposure results either in decrease of polyP formation rate or in prevalence of the processes of its consumption, thus lowering the content of polymers in the cell [9].

The goal of this work was to study the effect of the protonophore uncoupler CCCP on the synthesis of separate polyP fractions during the growth of *S. cerevisiae* VKM Y-1173 on the media with different carbon sources and different O_2 contents.

MATERIALS AND METHODS

The subject of research was the yeast *Saccharomyces cerevisiae* VKM Y-1173. Cells were grown on a shaker at 29°C in flasks with the Reader medium [15] with 2% glucose as a carbon source under low, <0.1 mmol O_2/l min (hypoxia), or high, 0.56 mmol O_2/l min, aeration and under intensive aeration with 1% ethanol vol/vol [10].

PolyP synthesis was studied under hypercompensation conditions. For this purpose, the yeast was grown on a complete Reader medium up to the midexponential phase on different carbon sources under different aeration levels. Then the cells were aseptically transferred to the same media without P_i and grown for 7 h (starvation, –P). Thus-obtained cells were again transferred to the complete medium (+P) and further grown for 0.5 h in the absence of CCCP (control) or in the presence of different CCCP concentrations. The 0.5-h exposure was chosen because the maximum synthesis of all polyP fractions was observed during this period of growth. In the following period of growth, the accumulation of total polyP abruptly decreased and ceased at all for some fractions [9]. Moreover, it was shown that 0.5-h incubation with CCCP was sufficient for its maximum uptake by *S. cerevisiae* cells [16].

PolyP was isolated from the cells by the Langen–Liss method: successive extraction from cells at 0°C by acid, saline, and alkaline solutions. As a result, the following polyP fractions were obtained: acid-soluble (polyPI), salt-soluble (polyPII), and two alkali-soluble fractions (polyPIII at pH 9–10 and polyPIV at pH 12). The content of the acid- and alkali-insoluble fraction polyPV was assessed by orthophosphate formed after the treatment of residual biomass with 0.5 M $HClO_4$, 90°C, twice by 20 min [17].

ATP was extracted and assayed using the luciferin–luciferase preparation (Sigma, United States) in a 1250 luminometer (LKB, Sweden) as described [18].

The rate of O_2 uptake by intact cells was measured in a thermostatically controlled (29°C) polarographic cell (V = 2 ml) using a Clark-type platinum electrode enclosed in a Teflon membrane as described [19]. Phosphorous compounds, P_i, glucose, optical density (OD) and yeast biomass dry weight were measured by the known methods [17].

The presented experimental results were obtained in three statistically treated biological experiments [20].

RESULTS AND DISCUSSION

It has been shown that low CCCP concentrations (5–7 μM) decrease the gradient of H^+ ions across the vacuolar membrane in *S. cerevisiae* cells. Higher concentrations of this agent cause deenergization of the inner mitochondrial (10–15 μM) and plasma (>20 μM) membranes [13].

Based on the previous data showing that the synthesis of some polyP fractions correlates with the major energy transduction processes in cells [12], the effect of CCCP on polyP synthesis was studied against short-term (0.5 h) hypercompensation of these compounds in *S. cerevisiae* VKM Y-1173 grown on glucose under O_2 deficiency (hypoxia) or active aeration and on ethanol under active aeration [10].

The table presents the data on the inhibitory effect of CCCP on yeast growth. It can be seen that 10 μM CCCP inhibited growth on glucose under hypoxic and aerobic conditions compared to the control (hypercompensation, 0.5 h); the growth stopped completely in the presence of 25 μM CCCP in the medium.

The cells growing on ethanol were more sensitive to the protonophore: the growth was inhibited already at 1.5 and stopped completely at 5 μM CCCP.

Higher resistance of glucose-grown yeast to CCCP is supposed to be due to its simultaneous active release from the cells leading to a decrease in its actual intracellular concentration [16]. However, this assumption does not exclude the possible resistance of glycolysis to the uncoupler.

During growth on ethanol, when the basic energy-generating process in the yeast is oxidative phosphorylation at the level of the respiratory chain, due to the high sensitivity of mitochondria, low CCCP concentrations already result in the uncoupling of the energy-generating process (oxidative activity) and the energy-requiring processes (ATP synthesis and active transport) through dissipation of transmembrane $\Delta\mu H^+$ [21].

Indeed, as can be seen from Fig. 1, the highest rate of O_2 uptake under the action of CCCP was observed in the cells grown on ethanol. Under these conditions, O_2 uptake reached its maximum already at 2 μM CCCP and remained at a high level with up to 5 μM CCCP in the medium.

During the growth on glucose under high aeration, the maximum O_2 uptake was observed at 5 μM CCCP; under hypoxia, O_2 uptake reached this value only at 10 μM CCCP. At the same time, O_2 uptake was half that under high aeration.

It is known that promitochondria are prevalent in yeast cells during their growth on glucose and under hypoxia [22]. In *S. cerevisiae* VKM Y-1173 growing on glucose, both under hypoxia and under high aeration of the medium, the content of cytochromes is at least 50% of the value in the cells growing on ethanol. This suggests rather high activity of mitochondria under the given cultivation conditions [10]. Such a conclusion was drawn also on the basis of ^{31}P-NMR analysis of the yeast growing on glucose under active aeration of the medium [13].

Considering these data and the fact that the growth rate of *S. cerevisiae* VKM Y-1173 on glucose under aeration is higher than under hypoxia [10], this strain can be referred to the yeasts with the lowered Crabtree effect [23]. The differences in O_2 uptake depending on culture growth conditions are noteworthy. They probably result from the fact that O_2 acceptance under active aeration occurs mainly due to the respiratory chain. Under hypoxia, predominant O_2 acceptors are the processes associated, in particular, with the synthesis of sterols and unsaturated fatty acids. It has been shown that hypoxia increases expression of a number of genes participating in these processes, as well as cytochromes b_5 and P450 of the microsomal fraction [24].

Figure 2 presents the results of studying the content of P_i, ATP, and total polyP in yeast cells under experimental conditions.

These data demonstrate that CCCP in the concentrations used in the experiment had almost no effect on the level of intracellular P_i during growth on glucose under hypoxic conditions. Under high aeration of the medium, at lower P_i background level in the cells, its content did not depend on CCCP concentration either.

Effect of CCCP on the growth (OD_{530}) of the yeast *S. cerevisiae* VKM Y-1173 during cultivation on different carbon sources under hypercompensation

Experimental conditions	2% glucose, hypoxia	2% glucose, aeration	1% ethanol (vol/vol), aeration
Initial culture, $-P_i$, 7 h	2.09	2.06	1.53
Hypercompensation, $+P_i$, 0.5 h			
Control	2.28	2.36	1.71
0.5 μM CCCP	2.28	2.36	1.70
1.5 μM CCCP	2.28	2.36	1.58
5 μM CCCP	2.27	2.34	1.53
10 μM CCCP	2.21	2.32	–
25 μM CCCP	2.09	2.07	–

The same picture was observed during growth on the medium with ethanol.

Thus, the results showing the resistance of P_i level to the uncoupler confirm the great significance of stable P_i content for yeast metabolism [25].

The ATP content significantly varied depending on the cultivation conditions. It was highest in the cells growing on ethanol and lowest in the yeast grown on glucose under hypoxic conditions (Fig. 2). These results confirm our previous data on the higher energetic efficiency of oxidative phosphorylation at the level of the respiratory chain compared to glycolysis [12]. In the presence of CCCP, the ATP level dropped by 50% during the cultivation on glucose under hypoxia at the protonophore concentration that still had no effect on culture growth (table).

On the contrary, during culture growth on the medium with ethanol, the ATP level decreased by 50% only at the CCCP concentration inhibiting yeast growth. It is interesting that the cells grown on the

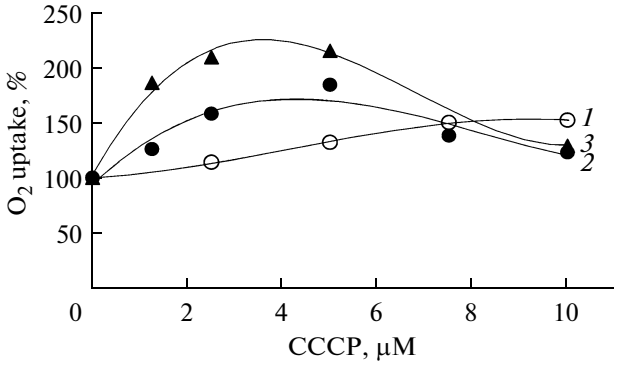

Fig. 1. O_2 uptake in the presence of CCCP by cells of the yeast *S. cerevisiae* cultivated on glucose under hypoxia (*1*) or aeration (*2*) and on ethanol (*3*) expressed in percent. One hundred percent corresponds to the value of O_2 uptake by cells in the absence of protonophore.

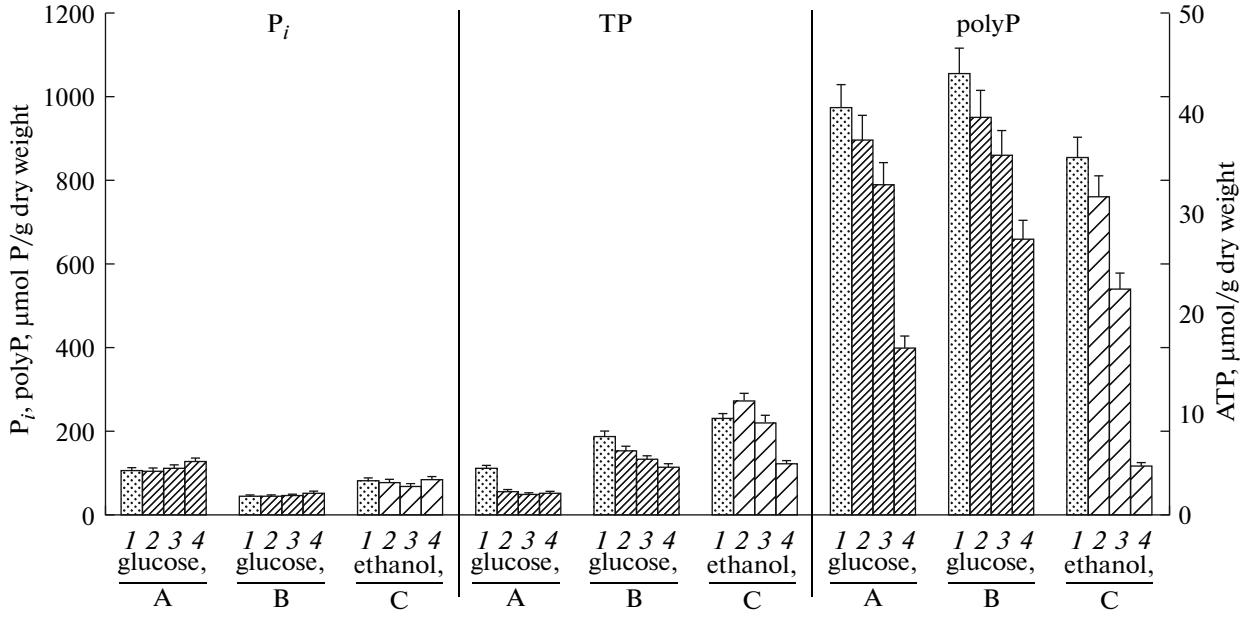

Fig. 2. Effect of CCCP on the levels of P_i, ATP, and polyP synthesis under hypercompensation conditions in the yeast growing on glucose under hypoxia (A), under aeration (B), and on ethanol (C); control cells (*1*); in the presence of protonophore: 5 μM CCCP for glucose and 0.5 μM CCCP for ethanol (*2*); 10 μM CCCP for glucose and 1.5 μM CCCP for ethanol (*3*); 25 μM CCCP for glucose and 5 μM CCCP for ethanol (*4*).

medium with glucose under high aeration proved to be more resistant to the uncoupler.

Unlike ATP, lower levels of polyP were accumulated in the cells growing on ethanol compared to those growing on glucose (Fig. 2). These data confirm the previous results on the absence of direct relationship between ATP content and polyP accumulation, which assumes the involvement of other energy sources in the synthesis of these polymers, probably due to $\Delta\mu H^+$ formed on the membranes [11, 12].

Our results show (Fig. 2) that a decrease in the level of polyP accumulation depended on CCCP concentration and was stepwise, independent of the cultivation conditions.

During the growth on glucose under hypoxia, the maximum CCCP concentration caused a 60% inhibition of polyP synthesis; during the growth on ethanol, polyP accumulation was inhibited by 80%.

The results of studying the effect of CCCP on the synthesis of different polyP fractions are presented on Fig. 3. It can be seen that conditions of *S. cerevisiae* cultivation substantially influenced the synthesis of individual polyP fractions. The low-molecular acid-soluble polyPI fraction was synthesized most actively during the growth on glucose under hypoxia (Fig. 3a). The synthesis of alkali-soluble polyPIII was predominant in the cells grown on glucose under active aeration (Fig. 3b). Finally, two fractions were actively synthesized during growth on ethanol: polyPIII and polyPV (Fig. 3c); synthesis of the latter during growth on glucose was substantially lower.

These data confirm the previous results demonstrating that the synthesis of polyPI is more closely associated with glycolysis, while the synthesis of polyPV is, rather, associated with oxidative phosphorylation at the level of the respiratory chain [9].

Low CCCP concentrations (5 and 10 μM) resulting in deenergization of the vacuolar membrane [13] predominantly inhibited the synthesis of polyPI and polyPIII fractions in the cells grown on glucose under hypoxia (Fig. 3a). At a CCCP concentration of 10 μM, the synthesis of polyPI and polyPIII decreased by 20 and 30%, respectively; at the same time, the protonophore had a weak inhibitory effect on the synthesis of other polyP fractions. During growth on glucose under active aeration, 10 μM CCCP decreased the synthesis of polyPIII and polyPV by 30 and 60%, respectively. The synthesis of other fractions was more resistant to this concentration of the uncoupler (Fig. 3b).

High CCCP concentration (25 μM), which disturbed the proton gradient across all cellular membranes, substantially inhibited the synthesis of all polyP fractions during yeast growth on glucose.

In the cells of *S. cerevisiae* grown on ethanol, the synthesis of polyPIII and polyPV fractions proved to be most sensitive to low CCCP concentrations (Fig. 3c). CCCP at a concentration of 1.5 μM inhibited the synthesis of polyPIII and polyPV fractions by 55 and 35%, respectively.

Thus, the data presented demonstrate that CCCP primarily inhibited the most actively synthesized polyP fractions depending on growth conditions:

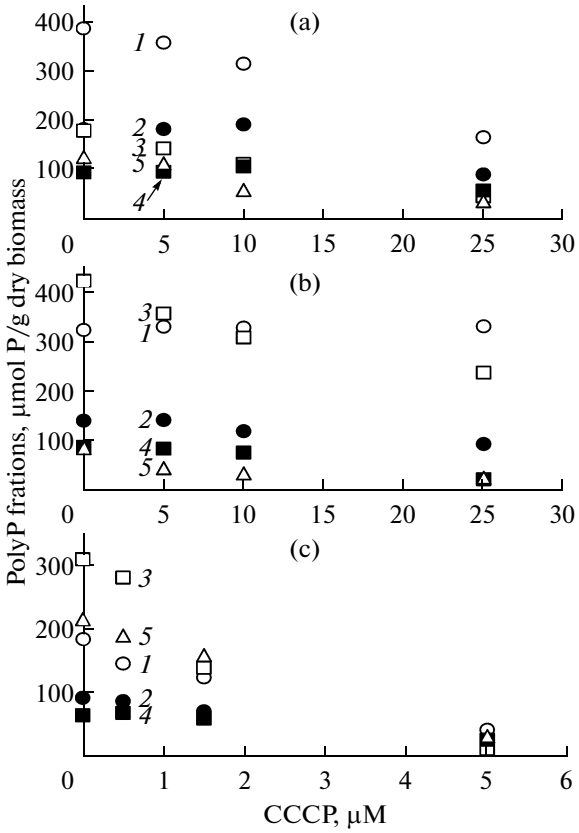

Fig. 3. Synthesis of individual polyP fractions in yeast cells depending on CCCP concentration in the medium during cultivation under hypercompensation conditions on glucose under hypoxia (a) or aeration (b) and on ethanol (c); fractions: polyPI (*1*), polyPII (*2*), polyPIII (*3*), polyPIV (*4*), and polyPV (*5*).

polyPI on glucose under hypoxia, polyPIII on glucose under aeration, and polyPIII and polyPV on ethanol.

Consequently, one might think that the synthesis of these very fractions is to a certain extent affected by the energetic state of the membranes. CCCP destroys the proton electrochemical potential $\Delta\mu H^+$ created on the vacuolar and plasma membranes by the work of proton pumps: V-ATPase and P-ATPase, respectively.

During yeast growth on ethanol, when the main energy source is oxidative phosphorylation at the level of the respiratory chain, CCCP destroys the proton gradient across the inner mitochondrial membrane and thereby inhibits the action of F_1F_0 ATP synthetase. Hence, polyP synthesis is inhibited.

Against this background, it is interesting that the synthesis of the salt-soluble fraction polyPII and the alkali-soluble fraction polyPIV proved to be most resistant to CCCP. It is probably due to the fact that the synthesis of polyPII in fungi is directly related to the synthesis of nucleic acids [26] and polyPIV formation is related to the synthesis of cell wall mannoproteins [8]; thus, these processes are less affected by the energetic state of the membranes.

The appropriate inhibitors and the P-ATPase and V-ATPase mutants will hopefully help differentiate the influence of activities of these proton pumps on polyP synthesis.

ACKNOWLEDGMENTS

The work was supported by the Russian Foundation for Basic Research (project no. 08-04-00472), Program of the Presidium of the Russian Academy of Sciences RAN P-24, and a grant for Support of Leading Scientific Schools of the Russian Federation (no. NS-1004.2008.4).

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