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EFFECTS OF ANOXIA ON ^{31}P NMR SPECTRA OF *PHYCOMYCES BLAKESLEEANUS* DURING DEVELOPMENT

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Abstract — The method of ^{31}P NMR spectroscopy was used to investigate the effects of anoxia on *Phycomyces blakesleeanus* mycelium during development. The greatest changes were recorded in the PPc, NADH, and α-ATP signals. Decrease of PPc signal intensity is due to chain length reduction and reduction in number of PPn molecules. Smaller decrease of β-ATP compared to α-ATP signal intensity can be attributed to maintenance of ATP concentration at the expense of PPn hydrolysis. Sensitivity to anoxia varies with the growth stage. It is greatest in 32-h and 44-h mycelium, in which PPn is used as an additional energy source, while the smallest effect was noted for 36-h fungi.

Key words: ^{31}P NMR, anoxia, *Phycomyces blakesleeanus*, development, polyphosphates

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INTRODUCTION

During development, fungi are often exposed to unfavorable growth conditions such as hypoxia or even anoxia. These environmental changes are bound to produce effects on cell energy metabolism, but such effects have been insufficiently studied. *Phycomyces blakesleeanus* is a strictly aerobic fungus (Ceredá-Olmedo and Lipson, 1987) and therefore can be a good model system for studying the effects of anoxia. Among methods that can be used, ^{31}P NMR spectroscopy is uniquely suited for such studies, since it permits nondestructive *in vivo* identification of phosphate compounds involved in energy metabolism.

When energy metabolism is considered, the ATP molecule is usually the first thing that comes to mind. For *Saccharomyces cerevisiae*, it was shown that ATP resonances in ^{31}P NMR spectra slightly decrease when yeast cells are transferred from aerobic to anaerobic conditions (den Hollander et al., 1981; Campbell-Burk et al., 1987). Intensity of the ^{31}P NMR signal of UDPG, which is a precursor for the synthesis of storage carbohydrates and cell wall material and thus an indicator of cell growth potential, shows no significant changes in *Candida tropicalis*, regardless of oxygenation (Lohmeier-Vogel et al., 1995).

In contrast to the signals of ATP and UDPG, major changes of ^{31}P NMR spectra during anoxia are observed in the signals of inorganic phosphate (P_i) and polyphosphates (PP_n) (Pilatus and Techel, 1991; Beauvoit et al., 1991). It is the general opinion that PP_n play an important role in fungal energy metabolism, since they regulate level of ATP and function as either a high-energy reserve or a phosphate reserve via hydrolysis (Harold, 1966). However, the effects of anoxia on intensity of PP_n signals are diverse. For *S. cerevisiae*, one group of results indicate that if the yeast cells are taken during the logarithmic growth phase, ^{31}P NMR spectra recorded in oxygenated and anoxic conditions show no significant differences (den Holander et al., 1981). However, if the yeast cells are taken during the stationary phase (when all glucose from the medium is used up), intensity of the PP_n signal decreases in anoxic conditions, while that of the intracellular inorganic phosphate (P_i) signal increases (den Holander et al., 1981; Campbell-Burk et al., 1987). A second group of results indicate that

in cells taken during the logarithmic phase, intensity of the PP_n signal also decreases in transition from oxygenized to anoxic conditions (Beauvoit et al., 1991). In *Neurospora crassa*, transition to anaerobic conditions in the presence of a carbon source does not lead to changes in intensity of the PP_n and P_i signals, while in a medium lacking a carbon source, the intensity of both signals slightly decreases (Pilatus and Techel, 1991).

Certain conclusions can be drawn from these results, in spite of their inconsistency. Transfer of yeasts to anaerobic conditions leads to synchronized changes in PP_n and P_i concentrations, and these changes depend on the development phase. The absence of a similar response in *N. crassa* may be a result of species specificity of respiratory chain structure, since a possible link between PP_n concentration and mitochondrial energy metabolism has been established (Beauvoit et al., 1989), together with considerable complexity and diversity of the "respiratory network" in fungi (Milani et al., 2001).

In our previous paper (Živić et al., 2007), we showed that changes of the PP_n to P_i signal intensity ratio are linked to characteristic stages of sporangiophore development. The obtained results suggested a role for polyphosphates as an energy and/or phosphate reserve during *P. blakesleeanus* development. In view of the contradictory results obtained on the effects of anoxia on ^{31}P NMR spectra in *S. cerevisiae* and *N. crassa* and their dependency on development, research on the effects of anoxia on *P. blakesleeanus* during different growth phases could provide an additional contribution to understanding fungal energy metabolism.

MATERIALS AND METHODS

Strains and growth conditions of mycelia

The wild type strain NRRL 1555(-) (Burgeff) of the fungus *Phycomyces blakesleeanus* was used in this study. One milliliter of spore suspension containing around 10^6 spores was seeded in standard minimal medium (Sutter, 1975): 36.7 mM KH_2PO_4, 2 mM $MgSO_4 \times 7H_2O$, 0.376 mM $CaCl_2$, 3 μM thiamine x HCl, 1 μM citric acid x H_2O, 3.7 μM $Fe(NO_3)_3 \times 9H_2O$, 3.5 μM $ZnSO_4 \times 7H_2O$, 1.8 μM $MnSO_4 \times H_2O$, 0.2 μM $CuSO_4 \times 5H_2O$, and 0.2 μM $NaMoO_4 \times 2H_2O$, but using doubled concentrations of glucose and L-asparagine (220 mM glucose, 26.6 mM L-asparagine) to ensure an adequate supply of carbon and nitrogen sources during growth.

Prior to seeding, the spore suspension was heat-shocked for 10 min at 49°C. Mycelia were grown in Petri dishes and stored in a growth cabinet under continuous overhead illumination with fluorescent light of 10 W/m^2 at 22°C and ca. 95% relative humidity.

NMR measurements

For NMR measurements, mycelia in specified growth phases (up to 10 Petri dishes were needed depending on the growth phase) were collected by vacuum filtration, washed with modified minimal medium (0.2 mM KH_2PO_4 without microelements, pH=4.65). An amount of 0.6-0.8 g (fresh weight) of mycelia was suspended in 2.5 ml of aerated modified minimal medium and packed in a 10-mm NMR tube. The mycelia were harvested every 4 hours from 16 h of growth onward in order to characterize the main growth stages. After recording of four control spectra, the samples were bubbled with N_2 for 5 min in order to replace the oxygenized atmosphere with an inert one. Four more spectra were taken in the oxygen-free atmosphere. The first (K_1) and fourth (K_4) control spectra, as well as the first (N_1) and fourth (N_4) spectra taken in anaerobic conditions, were used for analysis.

The ^{31}P NMR measurements were performed using a Bruker MSL 400 spectrometer operating at 161.978 MHz for ^{31}P. Spectra were accumulated in 4K data points with 7-μs pulse duration and 300-ms recycle time using a spectral width of 11363 Hz. Line broadening of 25 Hz was applied before Fourier transformation. Methylene diphosphonic acid at 17.05 ppm relative to 85% H_3PO_4 was used as an external standard.

RESULTS

The effects of anoxia on the ^{31}P NMR spectra of 36-h and 44-h mycelium of *Phycomyces blakesleeanus*

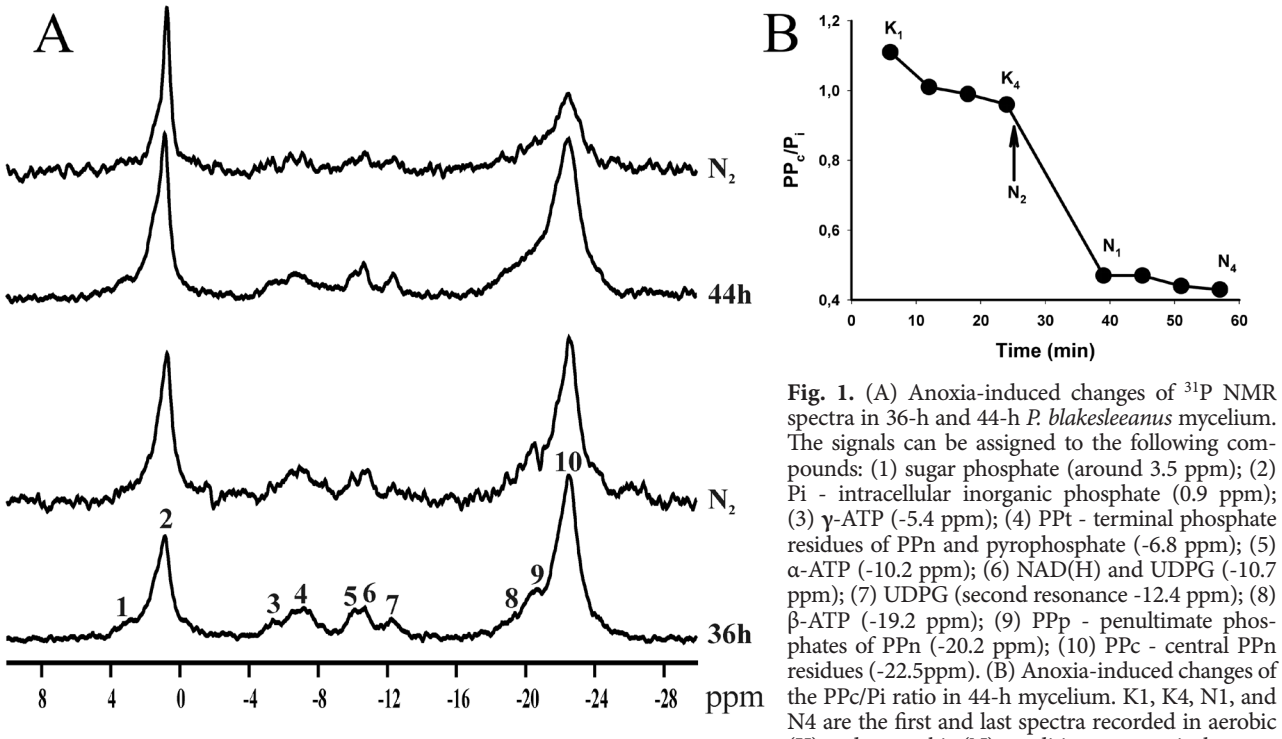

Fig. 1. (A) Anoxia-induced changes of ^{31}P NMR spectra in 36-h and 44-h *P. blakesleeanus* mycelium. The signals can be assigned to the following compounds: (1) sugar phosphate (around 3.5 ppm); (2) Pi - intracellular inorganic phosphate (0.9 ppm); (3) γ-ATP (-5.4 ppm); (4) PPt - terminal phosphate residues of PPn and pyrophosphate (-6.8 ppm); (5) α-ATP (-10.2 ppm); (6) NAD(H) and UDPG (-10.7 ppm); (7) UDPG (second resonance -12.4 ppm); (8) β-ATP (-19.2 ppm); (9) PPp - penultimate phosphates of PPn (-20.2 ppm); (10) PPc - central PPn residues (-22.5ppm). (B) Anoxia-induced changes of the PPc/Pi ratio in 44-h mycelium. K1, K4, N1, and N4 are the first and last spectra recorded in aerobic (K) and anaerobic (N) conditions, respectively.

are shown in Fig. 1. The assignment of signals was made as in a previous paper of ours (Živić et al., 2007). It can be seen that for 36-h spectra, noteworthy changes occur only in P_i signal intensity, which increases in anaerobic conditions by 31%, and in UDPG signal intensity, which decreases by 11%. Contrary to this, anoxia causes a large decrease in intensities of all signals in 44-h spectra except the P_i signal, whose intensity does not change. The largest decrease of intensity is observed for the PP_c signal (58%).

Figure 2 shows changes in ^{31}P NMR signal intensity ratios in control conditions (K_4/K_1), anaerobic and control conditions (N_1/K_4), and prolonged anaerobic conditions (N_4/K_4). Average signal intensity ratios for selected growth phases (Fig. 2A) and average signal intensity ratios of selected signals for all growth phases (Fig. 2B) are shown. In control conditions (K_4/K_1), only slight changes of signal intensity ratios (less than 10%) are observed, except for a statistically significant increase in P_i signal intensity of 17±5% and decrease in UDPG signal intensity of 21±2% (Fig. 2B).

Transfer to anaerobic conditions (N_1/K_4) causes decrease in intensities of all signals in the spectra except for P_i, whose intensity increases (10±7%, Fig. 2B). Decrease of intensities is statistically significant ($p<0.05$) for all signals except β-ATP and UDPG, the greatest declines occurring in the PP_c, α-ATP, and NAD(H) signals. Also, the decrease of α-ATP signal intensity (p=0,048) is greater than that of β-ATP signal intensity. Average signal intensity ratios for selected growth phases (Fig. 2A) are lower than in the control, except for 16-h specimens, which show a statistically significant intensity increase (13±4%), and 36- and 28-h specimens, which show no significant changes after N_2 treatment. The greatest decrease was obtained for 32- and 44-h fungi.

Prolongation of anoxia (N_4/K_4) results in further decrease of all signal intensity ratios except that of P_i, which remains constant. However, decrease in the signal intensity ratio is not statistically significant for the PP_c and NAD(H) signals. The effects of prolonged anoxia on ^{31}P NMR average signal intensity ratios for selected growth phases are most distinct at 16 h (p=0.005), 40 h ($p<0.001$) and 48 h

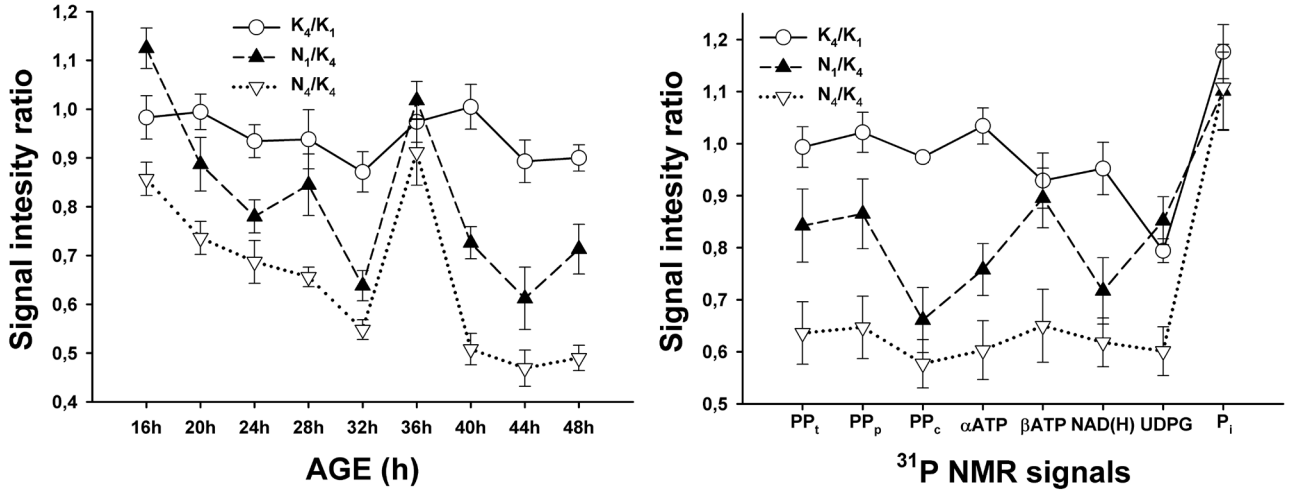

Fig. 2. Changes in the 31P NMR signal intensity ratio of *Phycomyces blakesleeanus* in control conditions (K4/K1, ○, black line), anaerobic and control conditions (N1/K4,▲, dashed line), and anaerobic conditions (N4/K4, ▽, dotted line). (A) mean values of all signal intensity ratios for selected growth phase; (B) mean values of intensity ratios of selected signal for all growth phases.

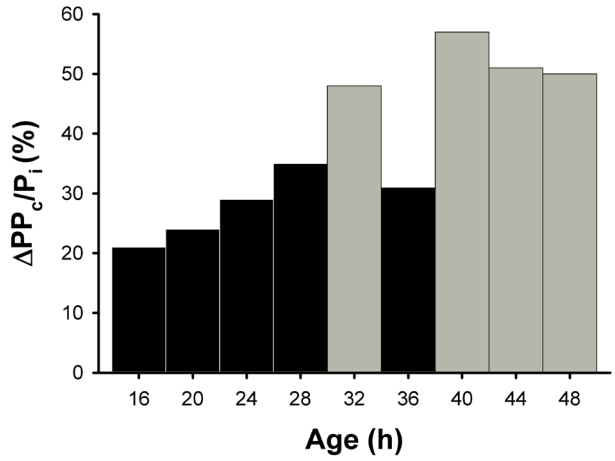

Fig. 3. Normalized changes of the PPc/Pi ratio in control (K4 spectrum) and N2-treated mycelia (N1 spectrum) in different growth phases.

(p=0.002) compared to N_1/K_4 values, while at 24, 36, and 44 h, prolonged anoxia has no statistically significant effect.

In *P. blakesleeanus*, changes in the PP_c/P_i ratio are linked to development phases (Živić et al., 2007). Since the PP_c signal shows the most pronounced intensity decrease during anoxia, while only the P_i signal increases, changes of the intensity ratio between these two signals were further analyzed in different growth phases during anoxia (Fig. 3). The results presented in Fig. 3 show that anoxia induces a decrease of the PP_c/P_i ratio, two groups dependent on the growth stage being discernible, the first (16-28 h) less sensitive, the second (32-48 h) more sensitive to anoxia. In the less sensitive group, the PP_c/P_i ratio varies from 21% (16 h) to 35% (28 h), while in the second group it ranges from 48% (32 h) to 57% (40 h), the only significant exception being recorded for 36-h fungi (31%).

DISCUSSION

It was confirmed earlier that PP_n play an important role in response to stress conditions like osmotic shock (Yang et al., 1993), changes in pH (Greenfield et al., 1987), and phosphate and glucose starvation (Bourne, 1991; Kulaev and Kulakovskaya, 2000). Having this in mind, it can be expected that PP_n will be involved in the response to a strong stress like transition to anaerobic conditions. However, research conducted to date did not firmly confirm these expectations. In *S. cerevisiae*, it was established that anoxia induces decrease of PP_c signal intensity and increase of P_i signal intensity in ^{31}P NMR spectra, but the intensity of those changes depends on the phase of growth yeast (den Hollander et al., 1981; Campbell-Burk et al., 1987; Beauvoit et al., 1991). In *N. crassa*, anoxia does not induce changes in intensi-

ties of these signals (Pilatus and Techel, 1991).

The herein obtained results indicate that anoxia leads to considerable changes in the ^{31}P NMR spectra of *P. blakesleeanus*, but the intensity of these changes varies as a function of both signal origin and development phase. The maximum decrease of intensity, recorded immediately after transition to anaerobic conditions, was observed for the PP_c signal. This was expected, considering that in various stress conditions like changes in medium pH (Greenfield et al., 1987) or osmolarity (Yang et al., 1993), one of the main homeostatic mechanisms is intensive polyphosphate hydrolysis. Decrease in the PP_c signal is greater than decrease in the PP_t signal, so it can be assumed that in this phase of PP_n hydrolysis, apart from exopolyphosphatase activity, endopolyphosphatases also have important role. Endopolyphosphatase hydrolyzes inner phosphoanhydride bonds, cleaving triphosphates from the PP_n chain, thus creating a large number of very short PP_n chains (Kumble and Kornberg, 1996). This enzyme was found only in the vacuole (Kumble and Kornberg, 1996). Considering the fact that in stress conditions vacuolar PP_n is hydrolyzed first (Kulaev et al., 1999), it can be assumed that this initial and most intensive PP_n hydrolysis generally takes place in the vacuole. However, with prolonged anoxia, the PP_t signal decreases significantly (Fig. 2B). It is highly unlikely that short chains connect up into longer ones, so this phenomenon could be explained by cessation of endopolyphosphatase activity due to the lack of PP_n with a sufficiently long chain. Exopolyphosphatases continue to hydrolyze remaining chains by cleaving marginal phosphates, thus shortening PP_n without increasing their number. This intensive PP_n hydrolysis is not accompanied by increase of P_i signal intensity (Fig 2B). Since the intensities of all other signals in the ^{31}P NMR spectra also decline, it can be assumed that even in anoxic conditions, part of the P_i obtained by PP_n hydrolysis is used for synthesis. Synthesis of ATP may be one of the ways to use this P_i, at least immediately after transition to anoxic conditions. To be specific, the first spectra taken in anaerobic conditions show larger decline of the α-ATP relative to the β-ATP signal, pointing to an increase of the ATP/ADP ratio. This could mean that the cell is trying to sustain ATP concentration at the expense of ADP, providing the means for maintaining basic cellular processes. Since *P. blakesleeanus*, unlike most fungi, is exclusively an aerobic organism (Galland and Russo, 1979; Wood-Baker, 1955), it is possible that the energy for ATP synthesis is obtained by means of polyphosphate kinase or AMP-phosphotransferase, which can catalyze ATP formation at the expense of PP_n (Kornberg, 1995; Bonting et al., 1991). This assumption is supported by the fact that polyphosphate kinase activity was registered in the vacuolar membrane, where most of the PP_n hydrolysis takes place in this first stage. Moreover, in prolonged anaerobic conditions, when the intensity of hydrolysis decreases, the difference in decline of intensity of the α- and β-ATP signals is lost.

Regarding growth phases, 36-h fungi are least sensitive to anoxia, while 32-h and 44-h fungi are most sensitive (Fig 2A). It has been demonstrated that 32-h and 44-h fungi use PP_n as an additional source of energy (Živić et al., 2007). It is then understandable that these growth phases are most affected by anoxia, given that they are most energy-demanding. The lack of changes in the spectra of 36-h specimens is much harder to explain. In this spectrum, only two signals change significantly: P_i signal intensity increases, while UDPG signal intensity decreases. However, these two signals show similar changes in control conditions, except that in the control, the change of P_i signal intensity is smaller, while that of UDPG signal intensity is greater. This means that part or all of the change in these signals is not necessarily induced by anoxia, but by some other, still unknown mechanism.

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ИСПИТИВАЊЕ ЕФЕКАТА АНОКСИЈЕ НА ^{31}P NMR СПЕКТАР ГЉИВЕ *PHYCOMYCES BLAKESLEEANUS* ТОКОМ РАЗВИЋА

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За испитивање ефеката аноксије на мицелијум гљиве *Phycomyces blakesleeanus* коришћен је метод ^{31}P NMR спектроскопије. Највеће промене су забележене код PP_c, NADH и α-ATP сигнала. Пад интензитета сигнала PP_c се може приписати смањењу дужине ланаца и смањењу броја PP_c молекула. Мањи пад интензитета сигнала β-ATP у поређењу са α-ATP се може објаснити одржавањем концентрације ATP-а на рачун хидролизе PP_c. Степен осетљивости на аноксију зависи од фазе раста, највећи је код 32 и 44 сати старог мицелијума који користи PP_c као додатни извор енергије, а најмањи код 36 сати старих гљива.