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EFFECT OF LONG-TERM CYANIDE EXPOSURE ON CYANIDE-SENSITIVE RESPIRATION AND PHOSPHATE METABOLISM IN THE FUNGUS *PHYCOMYCES BLAKESLEEANUS*

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Abstract - The effects of long-term exposure (5 h) of *Phycomyces blakesleeanus* mycelium to 5 mM KCN on respiration and phosphate metabolites were tested. Exposure to cyanide, antimycin A and azide lead to a decrease in the activity of cyanide-sensitive respiration (CSR), and the ratio of core polyphosphates (PPc) and inorganic phosphates (Pi), which is a good indicator of the metabolic state of a cell. After 5 h of incubation, the activity of CSR returned to control values. For this, the recovery of cytochrome c oxidase (COX) was required. In addition, the PPc/Pi ratio started to recover shortly after initiation of COX recovery, but never reached control values. This led us to conclude that the regulation of polyphosphate (PPn) levels in the cell is tightly coupled to respiratory chain functioning. In addition, acutely applied cyanide caused two different responses, observed by ^{31}P NMR spectroscopy, that were probably mediated through the mechanism of glycolytic oscillations, triggered by the effect of cyanide on mitochondria.

Key words: *Phycomyces blakesleeanus*, cyanide sensitive respiration, polyphosphates, sugar phosphates, ^{31}P NMR

INTRODUCTION

It is generally thought that polyphosphates (PPn) play an important role in fungal energy metabolism since they regulate the level of ATP and function as either a high-energy reserve or a phosphate reserve via hydrolysis (Harold, 1966; Kulaev and Kulakovskaya, 2000). Furthermore, ^{31}P NMR investigations of *Phycomyces blakesleeanus* showed that the ratio of core polyphosphate (PPc) and inorganic phosphate (Pi) signal intensities (PPc/Pi) can be a good indicator of the metabolic state of the cell (Živić et al., 2007); under anoxic conditions, it is reduced significantly (Stanić et al., 2009, 2013). It is to be expected that the inhibition of respiratory chain activity would also decrease the PPc/Pi ratio. However, our previous research (Zakrzewska et al., 2005) has shown that cyanide, a common blocker of cytochrome c oxidase (COX), unlike azide, actually increases this parameter.

Cyanide-resistant respiration (CRR), performed by alternative oxidase (AOX), is present in all plants and most fungi (Joseph-Horne et al. 2001). It is well known that in fungi the inhibition of the cytochrome pathway by cyanide or antimycin A can increase CRR capacity (Sherald and Sisler 1970, 1972; Sakajo et al. 1993), and such behavior was shown for *P. blakesleeanus* after incubation with antimycin A (Živić et al. 2009). However, comparable research

was not performed with cyanide, whose target is cytochrome c oxidase (COX), instead of Complex III. It was shown by Schubert and Brill (1968) that there can be a recovery of COX function after cyanide application. In addition, our previous work (Zakrzewska et al., 2005) has shown that cyanide may express some specific effects compared to other conventional inhibitors of cytochrome respiratory chain, like azide and antimycin A.

Having in mind the ubiquitous application of cyanide as an inhibitor of respiration, it is very important to test the complexity of its effects. In order to accomplish this, we have simultaneously measured the effects of three common respiratory inhibitors, cyanide, antimycin A and azide, on ^{31}P NMR spectra and respiration of *P. blakesleeanus* during prolonged periods of incubation.

MATERIALS AND METHODS

Organism, growth conditions and sample preparation

The wild-type strain of the fungus *Phycomyces blakesleeanus* (Burgeff) (NRRL 1555(-)) was used in this study. Spores (10^6) were cultivated in liquid standard minimal medium (Sutter, 1975) supplemented with 220 mM glucose and 26.2 mM L-asparagine, pH = 4.5 (Martinez-Cadena et al., 1995). Prior to inoculation, the spore suspension was activated by heat for 10 min at 49 °C. The mycelium was grown in Petri dishes placed in transparent plastic boxes and stored in the growth cabinet with continuous overhead white fluorescent light of 10 W/m^2, at a temperature of 22°C and ca. 95% relative humidity. For incubation experiments, the mycelia were incubated in 5 mM cyanide or 20 μM antimycin A for up to 5 h.

Measurement of oxygen consumption

Oxygen consumption was measured with a Clark-type oxygen electrode (Qubit Systems Inc. Kingston, Ontario, Canada). The mycelial suspension was diluted in fresh liquid nutrient medium to an appropriate concentration, and aerated for 2 min prior to the measurement of oxygen uptake. The initial concentration of the oxygen in the reaction buffer was ~260 nmol/ml. Two milliliters of mycelial suspension were transferred to a 4 ml electrode chamber and kept at a constant temperature (25°C). All measurements were performed at pH = 4.5. The inhibitors at the appropriate concentrations were applied directly to the electrode chamber containing the mycelial suspension; O_2 uptake was subsequently monitored for 10-15 min. Estimation of cyanide-sensitive respiration (CSR) and cyanide-resistant respiration (CRR) was performed by application of 1.5 mM KCN and 3 mM SHAM, respectively.

NMR measurements

For the NMR measurements, *P. blakesleeanus* mycelium was collected by vacuum filtration and washed with a modified minimal medium (0.2 mM KH_2PO_4, without microelements, pH = 4.5). About 0.6 g (fresh weight) of mycelia were suspended in 2.5 ml of aerated modified minimal medium, and packed in a 10 mm NMR tube. ^{31}P NMR measurements were performed using the Apollo upgrade, Bruker MSL 400 spectrometer operating at 161.978 MHz for ^{31}P. Spectra were accumulated with 14 μs pulse duration (about 45°) and 300 ms recycle time. All other experimental details were performed as described previously (Živić et al., 2007). The accumulation time was 6 min, making the effect of anoxia negligible (Hesse et al., 2000). The 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.

Determination of cyanide concentration

The concentration of cyanide ions was determined argentometrically as previously described by Liebig (1851). *P. blakesleeanus* mycelium was incubated in 5 mM KCN, and the remaining concentration of the CN^- ion was determined after 5 h.

Statistical analysis

The samples were compared statistically using the t-test or Mann-Whitney rank sum test at a 5% level

of significance ($p < 0.05$), performed with the Sigma Stat program version 2. Data are presented as means ± SEM with n indicating the number of independent experiments.

Chemicals

KCN, SHAM, azide and antimycin A were from Sigma-Aldrich (St Louis, MO, USA). All other reagents and chemicals were of analytical grade. KCN and azide were prepared using glass-distilled deionized water, and SHAM and antimycin A were dissolved in 96% ethanol.

RESULTS

Influence of respiratory inhibitors on CRR participation in total respiration and polyphosphate metabolism

Both antimycin A and cyanide are inhibitors of cytochrome respiration, but their targets are different: antimycin A inhibits Complex III while cyanide inhibits Complex IV (COX). It was shown previously that incubation in antimycin A increases the activity of cyanide-resistant respiration (Živić et al., 2009); here we compared the effects of long-term exposure (up to 5 h) of *P. blakesleeanus* mycelium to cyanide (5 mM) and antimycin A (20 μM).

Fig. 1A (white circles) shows the participation of CRR in total respiration after incubation in 5 mM KCN. During the first 100 min of incubation, alternative respiration was practically the only mode of respiration with participation of about 90% (20 min: 95.18±4.82%; 40 min: 99.32±0.68%; 60 min: 97.75±1.41%; 80 min: 91.51±3.27%; 100 min: 85.47±3.99%, n = 5 for all time points), since the cytochrome respiration was inhibited by cyanide. After this, CRR participation exponentially decreased, which started a little before the 100th min, slowed around the 150th min (120 min: 74.20±5.64%; 140 min: 55.02±3.81%; 160 min: 54.10±3.40%; 180 min: 51.01±2.97%, n = 5 for all time points), and ended at around 200 min of incubation when CRR reached stable values of about 50% of participation (200 min: 46.54±4.09%; 240 min: 47.43±4.07%; 260 min: 47.72±2.12%; 300 min: 41.76±4,42%, n = 5 for all time points). There was a statistically significant difference between the participation values at the 100th and 140th min ($P = 0.004$). After 100 min of incubation, respiration again became sensitive to cyanide, i.e., the participation of cyanide sensitive respiration (CSR) in total respiration increased (Fig. 1B, white circles). This increase is also shown in Fig. 1C (white circles) with the activities ($\mu molO_2\ min^{-1}\ g_{DW}^{-1}$) of CSR at various time points normalized to CSR activity in control. After 240 min of incubation, CSR activity reached the value of the control ($CSR_{240}/CSR_c = 1.045 \pm 0.008$, n = 5). When the *P. blakesleeanus* mycelium was incubated in antimycin A, there were no changes in CRR participation during incubation and therefore no CSR recovery (Fig. 1A and B, black circles). In this case, respiration remained cyanide insensitive and increased slightly compared to its value at the beginning of the incubation period, due to CRR induction in antimycin A (Fig. 1D, n = 3 for all time points). There was also a significant increase of CRR capacity during incubation in KCN (Fig 1C, black circles), but this increase ceased around 100th minute, which also marked the beginning of CSR recovery. When *P. blakesleeanus* mycelium was incubated in 5 mM NaN_3, results were very similar to those obtained with antimycin A (results not shown).

Since cyanide is very volatile in the acidic environment and *P. blakesleeanus* medium has a pH = 4.5, the possibility that the recovery of CSR is actually a consequence of cyanide evaporation had to be considered. To eliminate this possibility, KCN concentration in the medium after 5h of incubation was determined to be 1.29 mM, which is more than enough for inhibition of cytochrome respiration.

To link the effects of KCN and antimycin A on respiration with their effect on phosphate metabolism, we analyzed the changes of PPc/Pi signal intensities in ^{31}P NMR spectra after incubation with both inhibitors. PPc/Pi ratio changes during 5 h of incubation of *P. blakesleeanus* mycelium in 5 mM KCN are shown in Fig. 2A (black circles). At the beginning of the treatment, the PPc/Pi ratio decreased abruptly

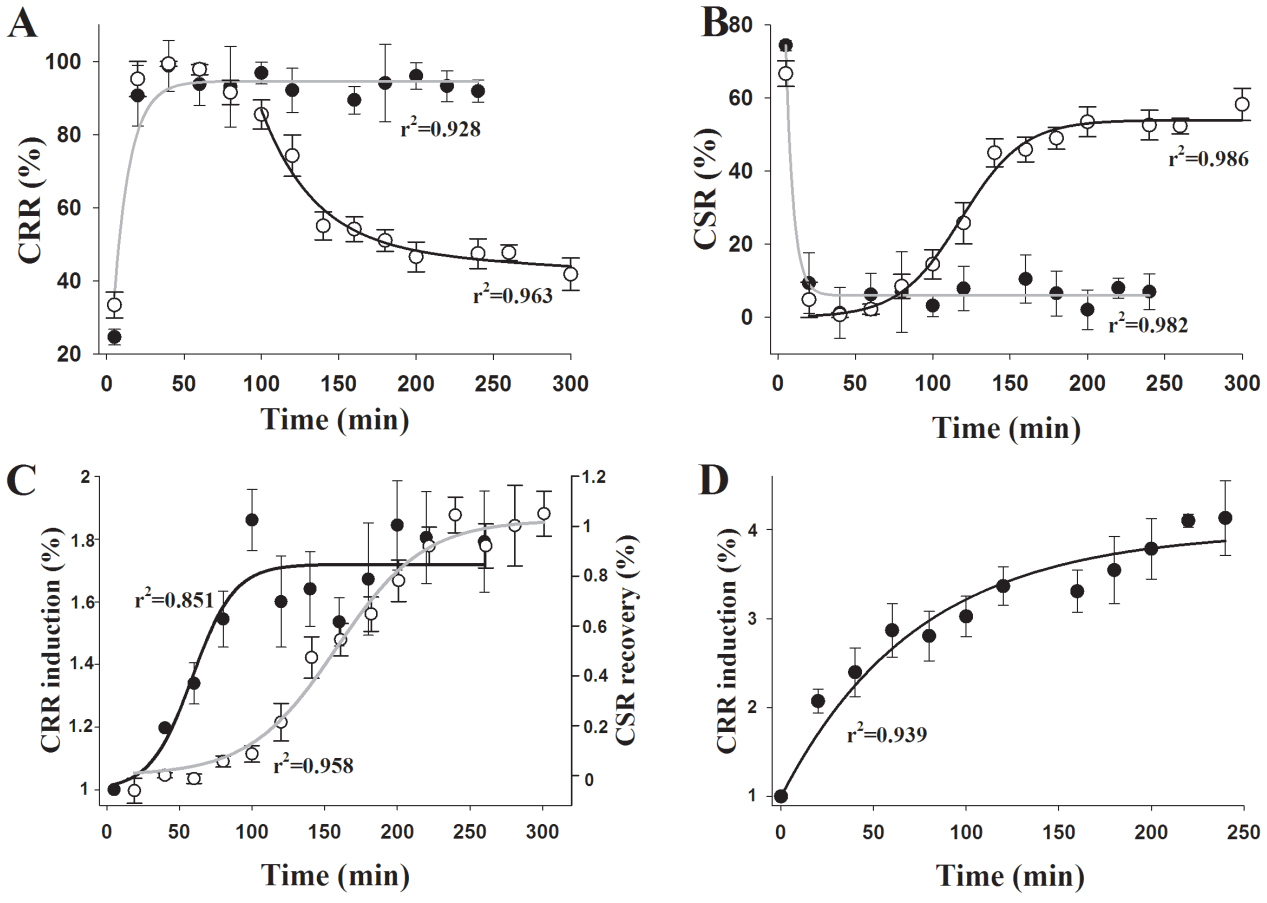

Fig. 1. A) CRR participation (%) in total respiration during 5 h incubation of *P. blakesleeanus* mycelium in 5 mM KCN (white circles, black line) and 20 μM antimycin A (black circles, grey line). B) CSR participation (%) in total respiration during 5 h of incubation in 5 mM KCN (white circles, black line) and 20 μM antimycin A (black circles, grey line). C) CRR induction during 5 h of incubation in 5 mM KCN shown as CRRn/CRRc (black circles, black line) and CSR recovery during 5 h of incubation in 5 mM KCN shown as CSRn/CSRc (white circles, grey line). CSR and CRR are expressed in $\mu molO_2\ min^{-1}\ g_{DW}^{-1}$. D) CRR induction during 4 h of incubation in antimycin A shown as CRRn/CRRc. Black and grey lines are fitted.

from 1.84±0.17 to 1.09±.07 at the 40th min, and to 1.01±0.01 at the 80th min, which was also a statistically significant minimum (P = 0.045). After that, the PPc/Pi ratio increased slowly (120 min: 1.15±0.07; 160 min: 1.17±0.08; 200 min: 1.19±0.06; 240 min: 1.28±0.07; n = 5 for all time points), but never returned to its control value. When we compared the results that describe the course of changes in the PPc/Pi ratio with those describing changes in CSR participation in total respiration (Fig 2A, white circles), recovery of the PPc/Pi ratio started just after CSR recovery, but unlike CSR, it never reached the control value.

When *P. blakesleeanus* was incubated in 20 μM antimycin A, there was no PPc/Pi recovery (Fig 2B). It decreased from 2.35±0.27 in the control to 1.58±0.06 within 5 min, and then further to 1.181±0.06 (20 min) and 1.09±0.02 (40 min, n = 5 for all assays). After that, the PPc/Pi ratio remained at values close to 1. When the *P. blakesleeanus* mycelium was incubat-

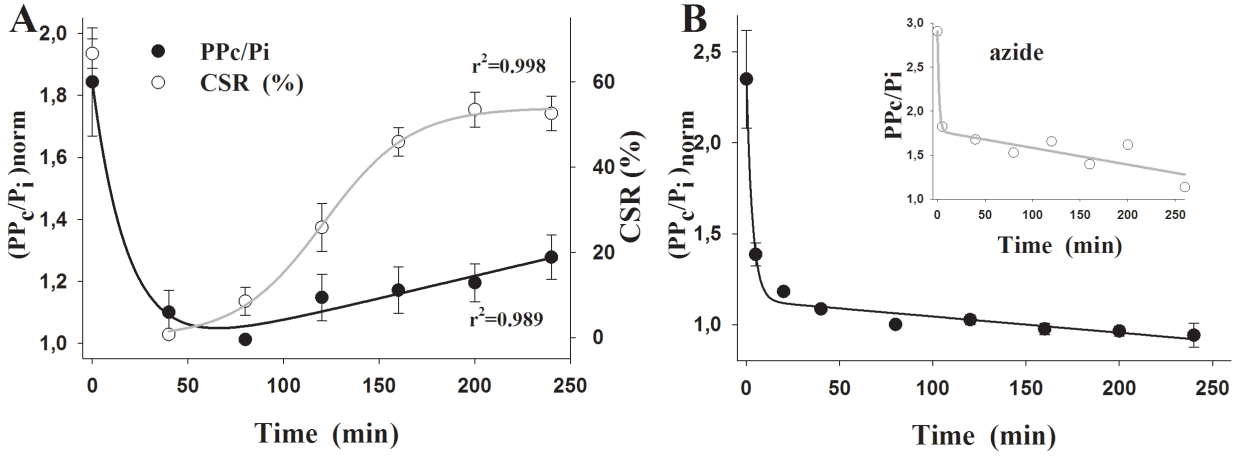

Fig. 2. A) PPc/Pi ratio during 5 h incubation of *P. blakesleeanus* mycelium in 5 mM KCN (black circles, black line – PPc/Pi ratio in every time point was normalized to minimal PPc/Pi.), and CSR participation in total respiration (white circles, grey line). B) Incubation in 20 μM antimycin A (black circles, black line – PPc/Pi ratio was normalized to PPc/Pi_{80min} which was minimal in experiments with cyanide) and 5 mM NaN_3 (insert). Black and grey lines are fitted.

ed in 5 mM NaN_3, results were very similar to those obtained with antimycin A (Fig. 2B, insert).

Influence of cyanide on ^{31}P NMR spectra of P. blakesleeanus

It was previously shown that, unlike azide whose application always induces a decrease in PPc and an increase in Pi signal intensities (decrease of PPc/Pi ratio), cyanide induces the opposite effect (Zakrzewska et al., 2005). However, this effect did not manifest in all subsequent experiments. To determine whether there is any time regularity in the occurrence of the observed opposite effects of KCN, control spectra and spectra with the addition of 10 mM KCN were recorded during 4 h or more. The results confirmed that cyanide has two different effects on the PPc/Pi ratio, where in the first (type I response), this parameter increased significantly compared to control, and in the second (type II response), it increased only slightly. These two effects alternated in time so that the effect of KCN on the PPc/Pi ratio seemed to be periodic (Fig. 3 A). To determine whether the effect of cyanide was indirectly caused by the inhibition of cytochrome respiration, the mycelium incubated in 20 μM antimycin A was used as a control, and 10 mM KCN was added every 20 min (Fig. 3C). The periodic effect of cyanide was not completely inhibited by antimycin A, indicating that it could be the result of its direct involvement in polyphosphate/phosphate metabolism. The same result was obtained after incubation of *P. blakesleeanus* mycelium in NaN_3 (results not shown).

Table 1 shows that cyanide has two different effects on the signals of other phosphate compounds in the ^{31}P NMR spectrum, with the most prominent effect on the sugar phosphate (SP) part of the spectrum, with increased SP signal intensities in the same spectra as the increase in the PPc/Pi ratio. The intensity changes of all signals are shown in Table 1 as $(I_{treatment}-I_{control})/I_{control}\times100$. Table 1 also shows changes in ^{31}P NMR spectra of *P. blakesleeanus* mycelium after the addition of 20 μM antimycin A.

The main changes in Type I response can be seen in the sugar phosphate (SP) part of the spectra. In the part of the spectra from 4 to 6 ppm (SP 1), the signal appeared only after the addition of cyanide (Fig 3B), while the intensity of the signal between 3 to 4 ppm (SP 2) increased significantly. In the Type II response to cyanide, the sugar phosphate signal in-

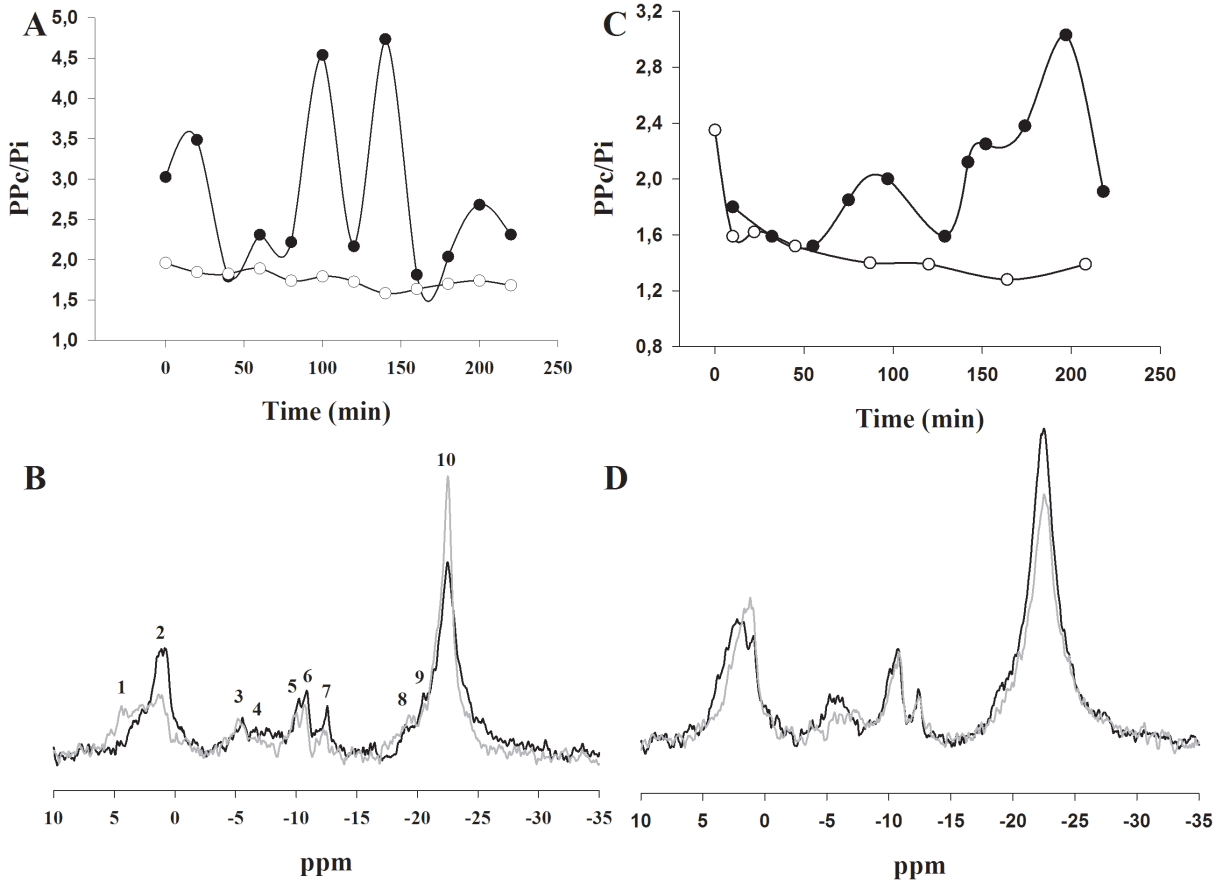

Fig. 3. The effect of 10 mM KCN on ^{31}P NMR spectra of *P. blakesleeanus* mycelium. A) Changes in PPc/Pi ratio after 10 mM KCN application compared to control during 5 h (white circles – control, black circles – 10 mM KCN). B) Representative spectrum of Type I response. The signals can be assigned to following compounds: 1 – sugar phosphates (SP), 2 – inorganic phosphates (Pi), 3 – γ ATP, 4 – terminal phosphate residues of PPn and pyrophosphate (PPt), 5 – α ATP, 6 – NAD(H) and UDPG, 7 – UDPG second resonance, 8 – β ATP, 9 – penultimate phosphates of PPn (PPp), 10 – central PPn residues (PPc) C) Changes in PPc/Pi ratio after addition of 10 mM KCN compared to mycelium incubated in 20 μM antimycin A (white circles - 20 μM antimycin A, black circles – 20 μM antimycin A + 10 mM KCN). D) Effect of 20 μM antimycin A on ^{31}P NMR spectrum of *P. blakesleeanus* mycelium. Black spectra- control, grey spectra – treatment.

tensity remained almost unchanged, while antimycin A induced its decrease (Table 1). In type I response, γ ATP and β ATP signals increased, while in Type II they decreased. Type I response is also distinct by the statistically significant decrease in ^{31}P NMR intensity of PPt, α ATP, NADH/UDPG and UDPG signals. A significant increase in PPc, and a decrease in PPt signals also occurred. This could mean that cyanide induced the lengthening of polyphosphate chains and the decrease in their numbers.

DISCUSSION

Cytochrome respiration recovery during P. blakesleeanus mycelium incubation in cyanide

Cyanide leads to the complete repression of cytochrome respiration in *P. blakesleeanus* mycelium immediately after application. However, this does not seem to be the case when the mycelium is incubated in 5 mM KCN. During the first 100 min of

Table 1. Changes in ^{31}P NMR signal intensities of *P. blakesleeanus* mycelium after the addition of 10 mM KCN and 20 μM antimycin A. Changes in signal intensities are shown as $(I_t\text{-}I_c)/I_c\times100$. The sign represents direction of the change. SP2 – sugar phosphate signal from 3 to 4 ppm. n – number of independent experiments, N – no changes compared to control, P – statistical significance (* - compared to type I response, † - compared to type II response). Symbols in P column represent significance of signal change compared to the same signal in the previous column.

	KCN		antimycin A	P
	type I	type II		
SP 2	28,06±4,55	N	-22,98±8,35	*;*†
Pi	-28,39±2,44	-8,95±1,93	44,54±13,13	*;*†
γATP	10,26±3,13	-5,90±3,19	-18,28±4,95	*;*
PPt	-27,16±2,75	-13,37±3,35	N	*;*
αATP	-17,23±2,42	-10,70±1,92	N	*;*†
NADH/ UDPG	-20,66±1,51	-9,16±1,60	N	*;*†
UDPG	-20,14±2,21	-14,44±2,12	N	*;*†
βATP	12,34±2,98	-12,93±2,28	N	*;*
PPc	31,24±6,72	-0,25±1,27	-10,63±2,19	*;*†
n	44	30	6	

incubation, almost all respiration measured could be attributed to alternative respiration. After this period, respiration sensitive to 1 mM KCN was slowly reestablished. After 5 h of incubation, the activity of CSR returned to the control values. The possibility that this effect is a consequence of cyanide evaporation was tested by measuring the concentration of the cyanide ion in the mycelium suspension after 5 h of incubation. Its concentration (1.29 mM) turned out to be enough for COX inhibition. The third type of respiration sensitive to 1 mM KCN and high concentrations of SHAM (10 mM), induced by incubation in antimycin A, was found in *P. blakesleeanus* (Živić et al., 2009), but since this concentration of inhibitor was already present in the mycelial suspension, the recovery of CSR cannot be attributed to its induction. The possibility of induction of additional terminal oxidases cannot be excluded, however, they would be different from those found in *Candida parapsilosis*, cytc_{PAR} and oxc_{PAR} (Milani et al., 2001; Guerin & Camougrand, 1994), since they are sensitive to high concentration of cyanide – 10 mM. The activity of alternative respiration during the period of complete repression of cytochrome respiration increased to a value 70% larger than that in untreated mycelium, which means that cyanide induces CRR just like antimycin A. However, at the beginning of CSR recovery, the increase in CRR ceased and the participation of CRR in total respiration decreased. Different behavior was observed during incubation of *P. blakesleeanus* mycelium in antimycin A. There was no recovery of CSR, participation of alternative respiration increased much faster and remained stable during the rest of the experiment. This leads to the conclusion that the recovery of cytochrome c oxidase, a direct target of KCN inhibition, is required for CSR recovery.

COX recovery was registered in mice, rat and gerbil liver. After delivery of a sub-lethal dose of KCN, COX recovered within 20 min or 1 h (Schubert and Brill, 1968), but this phenomenon was not thoroughly studied. Application of pyruvate in excess (Nůsková et al., 2010) was shown to lead to an incomplete recovery of COX, and the mechanism of this recovery is based on cyanohydrin formation. Pyruvate is the end-product of glycolysis and as such is ubiquitous in cells, however, as cyanohydrin formation requires pyruvate in large excess, this mechanism probably does not play a major role in COX protection from cyanide inhibition. Besides, CSR recovery takes place between 1.5 and 3 h of incubation and since the con-

centration of the CN^- ion was still high enough for COX inhibition after 5 h of incubation, the formation of cyanohydrin can be ignored here.

A possible mechanism of COX recovery during incubation of *P. blakesleeanus* mycelium in KCN is the release of a COX active site by nitrogen oxide, NO (Collman et al., 2008). CN^- binds to the oxidized form of heme a_3 (Fe^{3+}) with greater affinity than to the reduced form (Fe^{2+}), and shifts the reduction potential of heme a_3 from +350 mV to +150 mV, thus precluding any further reduction of cyt *c* (Kojima & Palmer, 1983). Although this CN^- complex is stable in O_2, it is readily reduced to the ferrous form in the presence of 1 equivalent of superoxide (Collman et al., 2008). An increase in O_2^- concentration was recorded in COX when heme a_3 was inhibited by various ligands (CN^-, CO) (Sipos et al., 2003), and it was produced *in situ* by COX via one electron reduction of O_2 by CuA. NO has a high affinity for the reduced form of heme a_3 and it can replace CN^- by forming a stable ferrous-nitrosyl complex, which can then be oxidized by O_2^-, regenerating the active enzyme (Collman et al., 2008). NO itself is also an inhibitor of COX, but this kind of "symbiotic interaction" between a potential inhibitor – NO, and a reactive oxygen species – O_2^-, could have a role in COX protection against external inhibitors. NO synthase (NOS), important for this process, was found in fungi and in *P. blakesleeanus* itself (Ninnemann and Maier, 1996; Maier et al., 2001). Leavesley et al. (2010) have shown that the inhibition of COX by KCN can be prevented by $NaNO_2$ pretreatment in dopaminergic N27 rat cells and isolated mitochondria. Nitrite application led to the increase of intracellular and mitochondrial NO concentration, but cyanide toxicity was reestablished by the addition of PTIO, a selective NO scavenger. Paradoxically, same authors (Leavesley et al., 2008) discovered in a different study that low NO concentrations produced endogenously by NOS increase the inhibition of COX by cyanide, while high NO concentrations induced by the addition of $NaNO_2$ to cultured cells have an antagonistic effect. Data on NO protective effect on COX were obtained mostly with mammalian cultured cells, isolated COX and by modeling, so the question remains as to how much of the stated can be applied to fungal metabolism, as there is almost no data available.

CSR recovery during the incubation of *P. blakesleeanus* mycelium in KCN was not observed during incubation with antimycin A. Since the target for antimycin A inhibition (Complex III) is different than that of KCN (COX), it is reasonable to assume that the defense mechanisms for these two inhibitors are different. On the other hand, incubation in azide, which has the same target place as KCN, shows similar effects as incubation in antimycin A. It is possibly a consequence of a different binding affinity of N_3^- to heme a_3 compared to CN^-, or of specific KCN effects.

The recovery of CSR is followed by the recovery of PPc/Pi ratio, which is somewhat slower than that of CSR. The PPc/Pi ratio does not recover after the initial fall when mycelium is incubated in antimycin A or azide, which further confirms that polyphosphate level is directly coupled to cytochrome respiratory chain activity (Stanić et al., 2013). When COX was inhibited by either chemical inhibitors or oxygen deprivation, the PPc/Pi ratio decreased, and after restoration of COX activity, the PPc/Pi ratio increased. During incubation in antimycin A and azide, respiration increased compared to the initial moment of inhibitor application, but this increase was caused solely by the increase in alternative respiration. Since there was no recovery of the PPc/Pi ratio, it is clear that the maintenance of PPn levels in the cell demands a fully functional cytochrome respiratory chain with proton pumping and ATP forming ability.

Effects of 10 mM cyanide on ^{31}P NMR spectra signal intensities

Even though there were no differences in mycelium cultivation or experimental setup, two different groups of KCN effect on ^{31}P NMR spectra could be clearly distinguished. The differences were most pronounced in the intensity changes of PPc and Pi signals (Fig 3, Table 1), hence their ratio (PPc/Pi)

in one group (type I response) grew abruptly, while in the second group (type II response) it grew only slightly compared to the control spectrum. During the 4 h of the experiment, these two responses to the treatment occurred alternately, giving the response of the PPc/Pi ratio a periodical nature. Apart from the PPc/Pi ratio, significant differences were noticed in the part of the spectrum containing signals of sugar phosphates, with signals at 4-6 ppm appearing only after cyanide addition, and those at 3-4 ppm increasing significantly. Glycolytic oscillations, i.e., oscillatory changes in the concentrations of glycolytic intermediates that are probably induced by oscillatory changes in the activity of phosphofructokinase (PFK) (Hess, 1979; Aon et al., 1991), can be connected to these changes in sugar levels in ^{31}P NMR spectra. Concentrations of glycolytic intermediates can vary up to two orders of magnitude during glycolytic oscillations (Hess, 1979), so these changes can be recorded with a relatively insensitive method such as ^{31}P NMR. Glycolytic oscillations were mainly studied in yeast (*Hess, 1979; Aon et al., 1991; Bier et al., 1996: Richard et al., 1994: Hald et al., 2012), they were noticed in rat brains (Shvets-Teneta-Gurii et al., 1998), and determined to have a significant role in insulin secretion from β-pancreatic cells (Merrins et al., 2012; Bertram et al., 2009). They are usually induced by the addition of cyanide after substrate (e.g. glucose), which is in agreement with our experimental conditions, since the mycelium was placed in an NMR tube with a fresh experimental medium containing glucose. According to the literature, the period of glycolytic oscillations varies from 1 to 20 min (Goldbeter, 1996), which is in agreement with the time frame of "oscillations" recorded in our experiments.*

Aon et al. (1991) have noted that all processes that increase cellular need for anaerobically produced ATP (such as respiration inhibition), and the changes of glucose influx to the system, increase the system's aspiration to start behaving in an oscillatory manner. The same authors noticed that the decrease in cytoplasmic ATP induces oscillatory behavior, but this decrease does not have to be a consequence of ATP synthesis inhibition, it can also be caused by the inhibition of the mitochondrial adenine nucleotide translocator. Long-chain acetyl-CoA-esters cause this inhibition, and their increase was recorded in mitochondria isolated from rat heart in the presence of KCN (Paulson and Shug, 1984). In these mitochondria, the activity of adenine nucleotide translocators was very low. However, in P. blakesleeanus spectra, ATP levels rose (Table 1) in type I response to cyanide, but the method that we used cannot point to the cellular location of ATP. The effect of cyanide on adenine nucleotide translocators could in part explain differences in the effect of cyanide and antimycin A on P. blakesleeanus spectra (Fig. 3, Table 1). In ^{31}P NMR spectra, 20 μM antimycin A significantly decreased the signal intensity of γATP, while other ATP signals did not change. In S. cerevisiae, antimycin A induced short-lasting and damped oscillations (Aon et al., 1991), so it is possible that even if this effect appeared in P. blakesleeanus, it could not be recorded by means of ^{31}P NMR spectroscopy. The decrease of transmembrane potential in mitochondria dampens the oscillations (Aon et al., 1991), so this can be used as an additional explanation for the differences in the effects of cyanide and antimycin A. Antimycin A targets Complex III of the respiratory chain, thereby excluding two proton translocating complexes, unlike cyanide, which targets Complex IV and excludes only one proton translocating complex of the inner mitochondrial membrane. In addition, Nůsková *et al., (2010) tested the effect of cyanide on COX affinity to oxygen, electron transport and transmembrane potential, and they concluded that KCN has greatest inhibitory effect on O_2 affinity, followed by electron transport and the smallest effect on transmembrane potential. We did not find data for any similar experiments performed with antimycin A.*

Adenine nucleotides have a significant role in the control of glycolytic oscillations, and their pool in the cell is balanced with PPn (*Kulaev i Kulakowskya, 2000*). An increase in the sugar phosphate signals occured in the same spectra where the PPc/Pi ratio also increased, so it can be assumed that the change in adenine nucleotide concentration after the addition of cyanide regulates both glycolytic oscillations and PPc/Pi ratio; it is possible that these two processes are also interdependent in some other, still unknown, manner.

It was shown, that a positive free enthalpy change occurs for an oscillating reaction compared with the negative free enthalpy change of the same reaction proceeding under non-oscillatory conditions with the same average values of the chemical potential of reactants and products (Durup, 1979). Thus, it might be significant and energetically advantageous for a system to run within the oscillatory domain (Hess, 1979) under the conditions of inhibited respiratory activity.

From all of the above-mentioned, it is clear that the effect of cyanide on the ^{31}P NMR spectra of P. blakesleeanus mycelium is probably achieved by its effect on mitochondria; the differences between KCN and antimycin A effects can be attributed to the subtle variations caused by different targets and modes of action.

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