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EXPERIMENTAL ARTICLES

Activation of the Alternative Oxidase of *Yarrowia lipolytica* by Adenosine Monophosphate

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Abstract—The study of the effect of nucleoside phosphates on the activity of cyanide-resistant oxidase in the mitochondria and the submitochondrial particles of *Yarrowia lipolytica* showed that adenosine-5'-monophosphate (AMP) did not stimulate the respiration of the intact mitochondria. The incubation of the mitochondria at room temperature (25°C) for 3–5 h or their treatment with ultrasound, phospholipase A, and the detergent Triton X-100 at a low temperature inactivated the cyanide-resistant alternative oxidase. The inactivated alternative oxidase could be reactivated by AMP. The reactivating effect of AMP was enhanced by azolectin. Some other nucleoside phosphates also showed reactivating ability, in the following descending order: AMP = GMP > GDP > GTP > XMP > IMP. The apparent reaction rate constant K_m for AMP upon the reactivation of the alternative oxidase of mitochondria treated with Triton X-100 or incubated at 25°C was 12.5 and 20 μM, respectively. The K_m for AMP upon the reactivation of the alternative oxidase of submitochondrial particles was 15 μM. During the incubation of yeast cells under conditions promoting the development of alternative oxidase, the content of adenine nucleotides (AMP, ADP, and ATP) in the cells and their respiration tended to decrease. The subsequent addition of cyanide to the cells activated their respiration, diminished the intracellular content of ATP by three times, and augmented the content of AMP by five times. These data suggest that the stimulation of cell respiration by cyanide may be due to the activation of alternative oxidase by AMP.

Key words: yeasts, mitochondria, cyanide-resistant alternative oxidase, regulation, activation by nucleotides.

Cyanide-resistant respiration is found in many higher plants, fungi, yeasts, and protozoa [1]. Such respiration is due to the functioning of a cyanide-resistant oxidase (also called alternative oxidase, or the alternative pathway), which transfers electrons from reduced ubiquinone (coenzyme Q) to oxygen independently of the main respiratory cytochrome-containing chain [1].

Alternative oxidase is located in the inner mitochondrial membrane [2] and is insensitive not only to cyanide but also to azide, CO, antimycin A, and myxothiazol [1]. On the other hand, this oxidase is specifically inhibited by benzohydroxamic acid (BHA) and its derivatives [3]. The alternative pathway branches from the cytochrome respiratory chain at the level of ubiquinone [4]. The alternative oxidase–mediated oxidation of the substrates that donate their electrons to ubiquinone (α-glycerophosphate, succinate, and exogenous NADH) is not coupled to phosphorylation [5]. However, the oxidation of NAD-linked substrates (such as pyruvate + malate) is associated with the synthesis of ATP by complex I. Moore and Siedow showed that alternative oxidase reduces oxygen with the formation of water but not hydrogen peroxide or superoxide radicals [6]. The affinity of the cyanide-resistant alternative oxidase for oxygen is considerably lower than that of cytochrome oxidase, as is evident from a comparison of their K_m values with respect to oxygen ($K_m > 1$ μM and $K_m < 0.1$ μM, respectively) [6].

The study of the mitochondria isolated from cyanide-resistant fungi and yeasts showed that their respiration can be resistant to cyanide. Further studies showed that alternative oxidase was present in the mitochondria but occurred in a latent state. The addition of the nucleoside monophosphates AMP and GMP activated the alternative oxidase [7–9].

The aims of this work were to study the ability of various nucleoside phosphates to activate alternative oxidase in yeast mitochondria and submitochondrial particles, to evaluate the intracellular content of adenine nucleotides in yeast cells (*Yarrowia lipolytica*) during the development of cyanide-resistant respiration, and to analyze how these parameters change in response to the action of cyanide and BHA.

MATERIALS AND METHODS

The yeast *Yarrowia lipolytica* strain VKM Y-155 used in this study was obtained from the All-Russia Collection of Microorganisms (VKM). The strain was grown in Reader medium containing (g/l) glucose, 10.0; $(NH_4)_2SO_4$, 3.0; $MgSO_4$, 0.7; $Ca(NO_3)_2$, 0.4;

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NaCl, 0.5; KH_2PO_4, 2.0; yeast extract (Difco), 2.0; and Burkholder trace element solution. The pH of the medium was 5.5–6.0. The strain was grown at 29°C in 750-ml Erlenmeyer flasks with 100 ml of the medium on a shaker (200 rpm).

To induce cyanide-resistant respiration, the exponential-phase yeast cells, which were sensitive to cyanide by 90–95%, were washed twice with distilled water, suspended in 10 mM phosphate buffer (pH 6.5) to a density of 3.0–3.5 mg dry wt/ml, and incubated at 29°C in a 750-ml flask with shaking at 200 rpm. The flask contained 25 ml of the cell suspension.

To isolate mitochondria, the stationary-phase yeast cells were washed twice with distilled water; suspended in 100 ml of 20 mM phosphate buffer (pH 6.0) containing 0.9 M sucrose, 1 mM EDTA, and 3% lyophilized enzyme preparation from snail gastric juice; and placed in a 750-ml flask. The flask was incubated at 29°C for 90 min with shaking at 200 rpm. Then the cells were washed twice with the same solution, but containing no enzyme preparation. The washed cells were suspended in a medium (referred to as the isolation medium) containing 0.5 M mannitol, 0.5 mM EDTA, and 0.1% bovine serum albumin in 10 mM Tris–HCl buffer (pH 7.2). The suspension was vortexed in a Biomix homogenizer (Hungary) at 14000 rpm for 2 min to disrupt cells. The cell homogenate was centrifuged two times at 3000 *g* for 10 min to remove unbroken cells and protoplasts. Mitochondria were precipitated by centrifugation at 7000 *g* for 30 min, resuspended in the isolation medium to a protein concentration of 10 mg/ml, and stored on ice.

To prepare submitochondrial particles (SMPs), the suspension of mitochondria was diluted fivefold with 50 mM Tris–phosphate buffer (pH 7.2) containing 0.5 mM EDTA and sonicated by using an MSE-150 ultrasonic disintegrator for a total of 2 min in 30-s bursts. SMPs were precipitated by centrifugation at 105000 *g* for 60 min, washed, and suspended in the same buffer.

The respiration of yeast cells, mitochondria, and SMPs was measured at room temperature (20–22°C) using a Clark-type oxygen electrode. The respiration medium (2 ml) was 10 mM Tris–phosphate buffer (pH 7.2) containing 0.6 M mannitol, 0.5 mM EDTA, and 0.1% bovine serum albumin. The initial concentration of dissolved oxygen in the medium was taken to be 250 μM.

To inactivate cyanide-resistant oxidase, the preparation of mitochondria was incubated at an elevated temperature (25°C) for 3–5 h or at a low temperature in the presence of phospholipase A (0.01 U/mg protein) or Triton X-100 at different detergent-to-protein proportions (from 0.05 to 0.5 mg/mg).

The suspension of azolectin was prepared by using a Potter homogenizer.

Adenine nucleotides were extracted from yeast cells with 5% perchloric acid. An aliquot (4.5 ml) of a culture was thoroughly mixed with 0.5 ml of 50% perchloric acid in a test tube, and the tube was placed on ice. Then the mixture was neutralized by adding 5 N KOH with vigorous shaking. The residue was removed by centrifugation or by passing through a paper filter. The extract was stored at –15°C. The concentrations of ATP, ADP, and AMP were determined by enzyme-linked reactions with hexokinase, glucose-6-phosphate dehydrogenase, pyruvate kinase, and myokinase [10]. The NAD(P)H produced in the reactions was assayed by using an MPF-4 fluorimeter (Hitachi, Japan).

The protein concentration in the suspensions of mitochondria and SMPs was determined with the biuret reagent. Aliquots of the suspensions were preliminarily dissolved in 1 N KOH containing 1% sodium deoxycholate.

RESULTS AND DISCUSSION

The representative curves illustrating the respiration of mitochondria isolated from cyanide-resistant yeast cells are presented in Fig. 1. Curve *1* shows that the freshly prepared yeast mitochondria oxidizing exogenous NADH exhibited relatively high values of the efficiency of phosphorylation (ADP/O = 1.7) and the respiratory control (RC = 2.3). In the absence of BHA, the oxidation of exogenous NADH was neither inhibited by cyanide nor activated by AMP (Fig. 1, curve *2*).

It has been previously shown that the incubation of mitochondria at 25°C for 3–5 h inactivates alternative oxidase [11] and makes the mitochondria unable to phosphorylate ADP. Such incubation also makes the oxidation of exogenous NADH sensitive to cyanide (Fig. 1, curve *3*). The subsequent addition of AMP to the mitochondria reactivated cyanide-resistant oxidase, as is evident from the complete inhibition of mitochondrial respiration by BHA. Azolectin enhanced the stimulating effect of AMP on alternative oxidase (Fig. 1, curve *3*) and could partially reactivate alternative oxidase when added alone (Fig. 1, curve *4*). A comparison of curves *3* and *4* shows that neither AMP nor azolectin, added separately, could completely restore the activity of alternative oxidase. This suggests that the inactivation of this oxidase may be due to two different processes, the impairment of the mitochondrial membrane and the exhaustion of the mitochondrial pool of nucleoside monophosphates.

Table 1 summarizes data on the effect of different nucleoside phosphates on the alternative oxidase of mitochondria incubated at 25°C for 5 h in the presence of azolectin. As can be seen from this table, 5'-AMP and 5'-GMP activated alternative oxidase to the greatest degree. The ability of the other nucleoside phosphates to activate alternative oxidase decreased in the following descending order: 5'-GDP > 5'-GTP > 5'-AMP > 5'-IMP > 5'-UMP. Four nucleoside phosphates (3'-GMP, 2'-GMP, 5'-ADP, and 5'-ATP) were unable to reactivate alternative oxidase. In general, the stimulating effect of

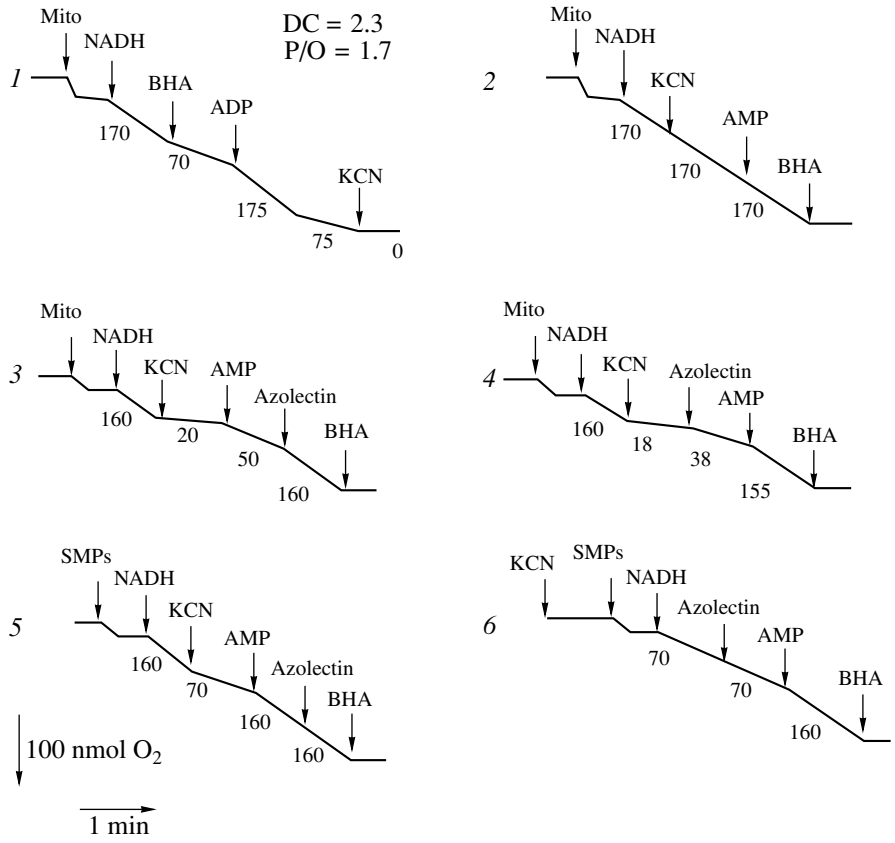

Fig. 1. The effect of 5'-AMP and azolectin on the respiration of *Y. lipolytica* mitochondria and submitochondrial particles (SMPs) oxidizing exogenous NADH in the presence of cyanide and the alternative oxidase inhibitor BHA: (*1*, *2*) intact mitochondria; (*3*, *4*) mitochondria after 5 h of incubation at 25°C; (*5*, *6*) SMPs obtained by the sonication of mitochondria (4 × 30 s). NADH, KCN, BHA, and AMP were added at concentrations of 1, 1, 5, and 1 mM, respectively. Numbers alongside of the curves indicate the oxygen consumption rate in nmol O_2/(min mg protein). "Mito" in all the figure legends denotes mitochondria.

5'-AMP and 5'-GMP on alternative oxidase can be considered to be specific.

The suppression of cyanide-resistant respiration was observed not only after the incubation of mitochondria at 25°C, but also after their treatment with ultrasound, phospholipase A, and Triton X-100 at a low temperature [11].

The oxidation of exogenous NADH by SMPs in the presence of cyanide was stimulated by AMP (Fig. 1, curves *5, 6*), although, as opposed to the alternative oxidase of intact mitochondria incubated at 25°C, the alternative oxidase of SMPs was not reactivated by azolectin. These data suggest that the treatment of mitochondria by ultrasound probably causes the loss of endogenous nucleoside monophosphates but does not alter mitochondrial membranes.

The incubation of mitochondria in the presence of the nonionic detergent Triton X-100 in a detergent-to-protein proportion of 0.3 completely inactivated alternative oxidase (Fig. 2, curve *1*). The addition of either azolectin (curve *2*) or AMP (curve *3*) to mitochondria partially reactivated alternative oxidase. The maximum reactivation of alternative oxidase was observed in response to the combined addition of azolectin and AMP to the mitochondria (curve *4*).

The incubation of mitochondria in the presence of phospholipase A for 20 min completely suppressed their cyanide-resistant respiration (Fig. 3, curve *1*). As in the case of Triton X-100 treatment, azolectin (Fig. 3, curve *2*) and AMP (Fig. 3, curve *3*) partially reactivated the alternative oxidase inhibited by phospholipase A. The maximum reactivation of the phospholipase A–inhibited alternative oxidase was observed when both azolectin and AMP were added to mitochondria (Fig. 3, curve *4*). These data provide further evidence that the complete inactivation of alternative oxidase is associated with impairment of the inner mitochondrial membrane and AMP loss.

Figure 4 shows the effect of 5'-AMP on the kinetic parameters of alternative oxidase in the mitochondria subjected to different treatments. The apparent K_m for AMP of the alternative oxidase of mitochondria treated with Triton X-100 or incubated at 25°C was found to be 12.5 and 20 μM, respectively (Fig. 4b). The apparent K_m for AMP upon the reactivation of the alternative oxidase of submitochondrial particles was 15 μM.

Table 1. The effect of various nucleotides on the activity of alternative oxidase in *Y. lipolytica* mitochondria

Nucleotide, 0.5 mM	Alternative oxidase, nmol O_2/(min mg protein)
Control	48 ± 3
5'-AMP	170 ± 9
5'-ADP	50 ± 3.5
5'-ATP	50 ± 3.5
5'-GMP	170 ± 10
5'-GDP	105 ± 8
5'-GTP	90 ± 7
3'-GMP	50 ± 4
2'-GMP	50 ± 4
5'-IMP	60 ± 5
5'-UMP	55 ± 4
5'-XMP	65 ± 6
5'-CMP	50 ± 5
3',5'-cAMP	50 ± 5

Note: The data are the means of three or four independent measurements. The reaction mixture contained 1 mM NADH, 1 mM KCN, 5 mg/ml azolectin, and 0.9 mg/ml mitochondria.

It should be noted that a detergent-induced decrease in the K_m of alternative oxidase for AMP (from 25 to 6 μM) was also observed by Vanderleyden *et al.* [8, 12], who suggested that, in the presence of detergents, mitochondrial alternative oxidases become more accessible to AMP.

Let us consider the data of Vanderleyden *et al.* in more detail. These researchers explained the ATP-induced decrease in the activity of alternative oxidase in *Moniliella tomentosa* mitochondria by the fact that ATP activates myokinase (adenylate kinase), which catalyzes the reaction ATP + AMP = 2ADP. This leads to a decrease in the AMP level and eventually to the inactivation of alternative oxidase. Taking into account that the inner mitochondrial membrane is impermeable to AMP [13] and that AMP is an activator of alternative oxidase, Vanderleyden *et al.* suggested that the AMP-sensitive component of alternative oxidase is located outside the inner mitochondrial membrane [8]. This suggestion contradicts our finding [2] that the alternative oxidase of higher plants and the yeast *Y. lipolytica* is located inside the inner mitochondrial membrane. In the latter case, it would be reasonable to suggest that alternative oxidase is controlled by the AMP level in the mitochondrial matrix. The observation that exogenous ATP does not cause any decrease in the activity of alternative oxidase (data not presented) can be accounted for by the location of adenylate kinase between the inner and outer mitochondrial membranes, which makes a rapid (in 1–2 min) decrease in the concentration of AMP in response to the addition of ATP improbable. If the activity of alternative oxidase depended on the concentration of AMP in the intermembrane space [8], the respiration of isolated mitochondria would be sensitive to cyanide, since the mitochondria must lose AMP in the process of their isolation (due to permeability of the outer mitochondrial membrane to nucleotides).

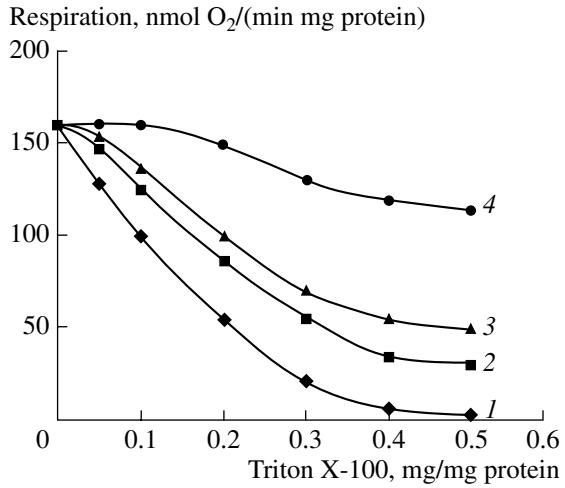

Fig. 2. The reactivating effect of AMP and azolectin on the alternative oxidase of mitochondria inactivated by different concentrations of Triton X-100. The curves show the respiration rate of mitochondria oxidizing NADH (1 mM) in the presence of (*1*) 1 mM KCN; (*2*) 1 mM KCN + 5 mg/ml azolectin; (*3*) 1 mM KCN + 1 mM AMP; and (*4*) 1 mM KCN + 1 mM AMP + 5 mg/ml azolectin.

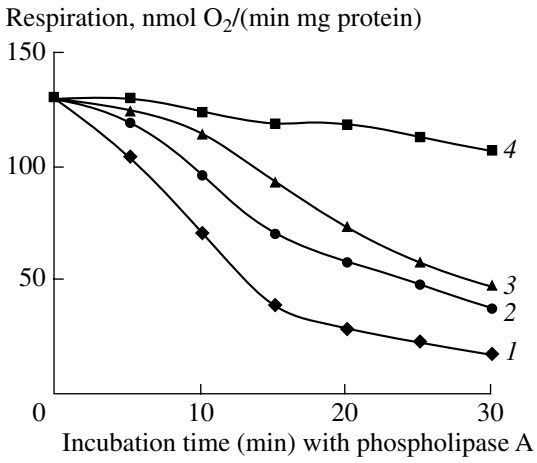

Fig. 3. The reactivating effect of AMP and azolectin on the alternative oxidase of mitochondria inactivated by phospholipase A (0.01 U/mg protein). The curves show the respiration rate of mitochondria oxidizing NADH (1 mM) in the presence of (*1*) 1 mM KCN; (*2*) 1 mM KCN + 5 mg/ml azolectin; (*3*) 1 mM KCN + 1 mM AMP; and (*4*) 1 mM KCN + 1 mM AMP + 5 mg/ml azolectin.

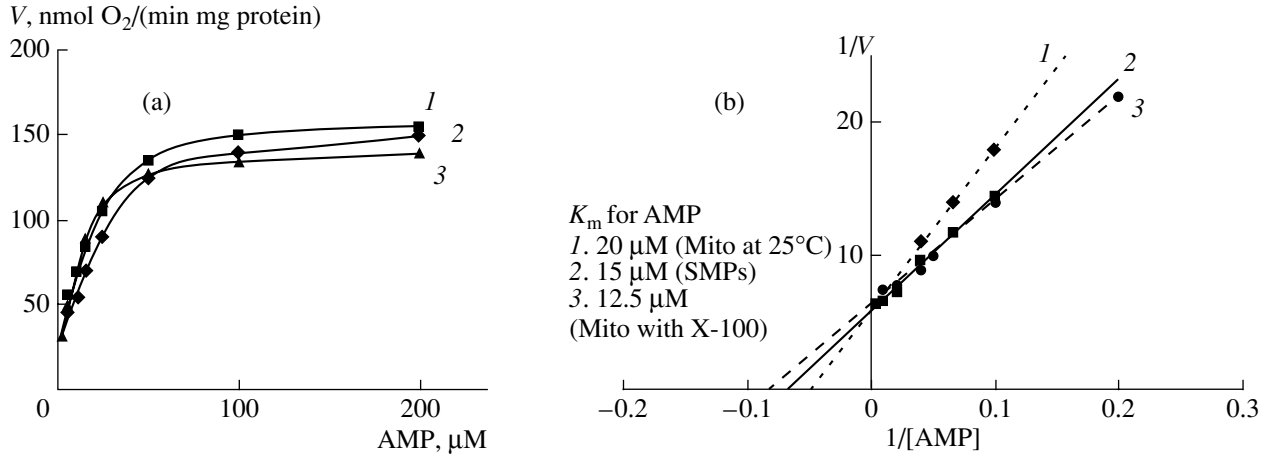

Fig. 4. (a) The reactivating effect of AMP on the alternative oxidase of mitochondria inactivated by (*1*) incubation at 25°C (5 h); (*2*) ultrasonic treatment (SMPs); and (*3*) incubation with Triton X-100 (0.25 mg/mg protein). The curves show the respiration rate of mitochondria oxidizing NADH (1 mM) in the presence of 1 mM KCN, 5 mg/ml azolectin, and different concentrations of AMP. (b) Lineweaver–Burk plot of the data presented in panel a.

This study showed that the inactivation of alternative oxidase is associated with the impairment of mitochondrial integrity. This implies that the inner mitochondrial membrane can become permeable to AMP. The associated decrease in the intramitochondrial concentration of AMP will inactivate alternative oxidase, whereas the addition of exogenous AMP must restore its activity.

It is likely that AMP is relatively tightly bound to the alternative oxidase of *Y. lipolytica* mitochondria, as is evident from the finding that it is only the detergent Triton X-100 that completely inhibited the alternative oxidase of these mitochondria (Fig. 2), while the other treatments induced only 15–20% inhibition (the incubation of mitochondria at 25°C for 3–5 h or in the presence of phospholipase A) (Fig. 3) or 50% inhibition (the sonication of mitochondria) (Fig. 1, curves *5*, *6*). These data imply that alternative oxidase loses AMP only after a treatment so severe that it causes a partial degradation of the inner mitochondrial membrane.

Table 2 shows changes in the level of adenine nucleotides in *Y. lipolytica* cells in the process of development of cyanide-resistant respiration. Initially, the respiration of the washed exponential-phase cells was highly sensitive to 1 mM cyanide (92% inhibition). The aerobic incubation of these cells in 50 mM phosphate buffer (pH 6.5) on a shaker (200 rpm) for 60 min diminished their endogenous respiration and sensitivity to cyanide about twofold (Table 2). After 90 min of incubation, the addition of cyanide not only failed to inhibit cell respiration, but even stimulated it. The maximum stimulation (by 87%) of cell respiration was observed after 2 h of incubation. The addition of BHA after cyanide inhibited cell respiration by 90% or more.

Table 2. The respiration and the adenine nucleotide content of *Y. lipolytica* cells during their aerobic incubation

Incubation conditions	Adenine nucleotide, nmol/mg dry biomass			Respiration, nmol O_2/(min mg dry biomass)			
	ATP	ADP	AMP	Control	1 mM KCN	5 mM BHA	1 mM KCN + 5 mM BHA
0 min	7.0 ± 0.7	2.10 ± 0.2	0.75 ± 0.08	105	9 (–92)	105 (0)	5 (–95)
0 min (+ KCN)*	0.2 ± 0.02	3.10 ± 0.3	3.20 ± 0.3				
30 min	5.0 ± 0.5	1.55 ± 0.2	0.65 ± 0.07	70	38 (–46)	70 (0)	3 (–96)
60 min	4.0 ± 0.4	1.30 ± 0.12	0.50 ± 0.06	55	50 (–9)	55 (0)	3 (–94)
90 min	3.5 ± 0.3	0.85 ± 0.1	0.40 ± 0.05	46	61 (+32)	44 (–5)	3 (–93)
120 min	3.3 ± 0.3	0.80 ± 0.1	0.40 ± 0.05	40	75 (+87)	38 (–5)	3 (–93)
120 min (+ KCN)*	1.0 ± 0.1	1.55 ± 0.2	2.05 ± 0.2				
120 min (+ BHA)*	3.35 ± 0.3	0.70 ± 0.1	0.40 ± 0.05				

Note: Parenthesized are the percents of inhibition of cell respiration by KCN and BHA (numbers with the minus sign). The plus sign indicates the stimulating action of cyanide on cell respiration. In the experiments marked by an asterisk (*), adenine nucleotides were extracted 5 min after the addition of an inhibitor.

During aerobic incubation, the content of adenine nucleotides in the cells gradually decreased (about twofold after 2 h of incubation). The addition of cyanide to the cyanide-resistant cells reduced the intracellular content of ATP by three times and increased the content of ADP and AMP by two and five times, respectively. In this case, as mentioned above, cyanide stimulated the respiration of these cells. In the absence of cyanide, BHA little influenced cell respiration and the content of adenine nucleotides (Table 2).

Our earlier studies showed that the alternative oxidase of *Y. lipolytica* is unable to compete with the cytochrome chain for electrons and can transfer to oxygen only the electrons that are excessive to the cytochrome chain [14]. This implies that the percent inhibition of respiration by BHA can measure the degree of involvement of alternative oxidase in cell respiration. The absence of the inhibitory action of BHA on cell respiration (Table 2) may have a twofold explanation. First, alternative oxidase may occur in a latent state (i.e., it may be potentially active but not involved in the transfer of electrons). Second, alternative oxidase may be inactive because of the low level of AMP in the mitochondrial matrix. In the latter case, the increase in the AMP level in the mitochondrial matrix must reactivate alternative oxidase. The observation that the addition of cyanide to the yeast cells considerably increased the AMP level (by five times) and cell respiration (Table 2) indicates that the second suggestion is more probable. That is, the activity of cyanide-resistant alternative oxidase in yeast cells is likely to be determined by the level of AMP available to the oxidase.

The activating effect of AMP on cyanide-resistant mitochondrial respiration was observed for the alga *Euglena gracilis* [7], the yeast *Hansenula anomala* [15], and the fungi *M. tomentosa* [8, 9, 12] and *Neurospora crassa* [16]. Unlike the alternative oxidase of yeasts and fungi, the alternative oxidase of higher plants is not activated by nucleoside monophosphates. On the other hand, it was found that the alternative oxidase of plants is activated by some α-keto acids, including pyruvate [17], and depends on the degree of reduction of disulfide bonds in the alternative oxidase molecule. Umbach *et al.* [18] showed that the alternative oxidase of plants occurs in the inner mitochondrial membrane as a dimer. In an oxidized state, the monomers are covalently bound by a disulfide bridge, whose reduction produces two sulfhydryl groups. The alternative oxidase of plants is four to five times more active in the reduced state than in the oxidized state [18].

Unlike the alternative oxidase of plants, microbial alternative oxidases are always monomers [19] and lack the cysteine residues that are involved in the activation of plant alternative oxidases by pyruvate [19, 20]. The mechanism of the stimulating effect of AMP on the alternative oxidase of yeasts is so far unknown. Based on the ability of AMP to activate the oxidation of duroquinol and ubiquinol-1 by mitochondrial membranes obtained from *N. crassa* and *Paramecium tetraurelia*, some researchers suggest that the favorable effect of AMP on the activity of alternative oxidase is due to enhancement of the interaction between the respiratory-chain quinones and a component of the alternative oxidase [15, 16].

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