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EXPERIMENTAL ARTICLES

Intracellular cAMP Content and the Induction of Alternative Oxidase in the Yeast *Yarrowia lipolytica*

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Abstract—The effect of cyanide, antimycin A, ethanol, and acetate on the induction of alternative oxidase in the yeast *Yarrowia lipolytica* VKM Y-155 was studied. The aerobic incubation of logarithmic-phase cells, whose respiration is sensitive to cyanide, in the presence of the aforementioned compounds led to the development of cyanide-resistant respiration, which could be suppressed by benzohydroxamic acid, an inhibitor of alternative oxidases. The incubation of cells with cyanide, ethanol, or acetate raised the intracellular pool of cAMP, which attained maximal values after a 2- to 3-min incubation period, then rapidly decreased to the initial value and did not change over the next three hours of incubation. The possible role of cAMP in the induction of alternative oxidase in yeast cells is discussed.

Key words: yeasts, alternative oxidase, cyanide-resistant oxidase, induction, cAMP content.

The cyanide-resistant respiration of higher plants, algae, fungi, yeasts, and protozoa is due to the functioning of a cyanide-resistant oxidase in mitochondria, also known as alternative oxidase or alternative electron transfer pathway [1]. Alternative oxidase is located in the inner mitochondrial membrane and shunts electrons from reduced ubiquinone to molecular oxygen, bypassing the main respiratory chain. Alternative oxidase is insensitive to cyanide, azide, antimycin A, and CO, but is specifically inhibited by hydroxamic acid derivatives, such as benzohydroxamic acid (BHA) and salicylhydroxamic acid (SHA) [1].

The alternative oxidase-mediated oxidation of the substrates whose dehydrogenases are located at the level of ubiquinone (α-glycerophosphate, succinate, and exogenous NADH) does not yield ATP [2], although the oxidation of NAD^+-dependent substrates through alternative oxidase is associated with ATP synthesis at the first site of oxidative phosphorylation (on the ubiquinone side of complex I) [2].

Alternative oxidase reduces molecular oxygen with the formation of water but not hydrogen peroxide or superoxide radicals [1]. The affinity of alternative oxidase for oxygen (K_m > 1.0 μM) is considerably lower than that of cytochrome oxidase (K_m < 0.1 μM) [1].

The alternative oxidase of higher plants and fungi is a 36- to 37-kDa protein encoded by chromosomal genes [1]. This alternative oxidase is induced by respiratory inhibitors, such as cyanide and antimycin A, and during growth on ethanol, acetate, and citrate [1, 3]. In plants, alternative oxidase is induced by salicylic acid [4], hydrogen peroxide, citrate, acetate, cysteine, and monofluoroacetate [5]. In yeasts, alternative oxidase is induced by oxidative and heat stress [6–8].

The origin of the intracellular signal responsible for the expression of the alternative oxidase gene has not yet been established, although the alternative oxidase gene of *Neurospora crassa* was found to contain a region sensitive to cAMP (the so-called cAMP-responsive element, CRE), which presumably controls the expression of the alternative oxidase gene [7].

The aim of the present work was to study the cAMP content of *Y. lipolytica* cells under conditions promoting the induction of alternative oxidase.

MATERIALS AND METHODS

The *Yarrowia lipolytica* VKM Y-155 strain, auxotrophic for thiamine, was obtained from the All-Russia Collection of Microorganisms (VKM).

The strain was grown in mineral Reader medium supplemented with 0.2% yeast autolysate, Burkholder trace element solution, and 1% glucose as the source of carbon and energy. The strain was cultivated at 29°C on a shaker (200 rpm) in 750-ml flasks with 100 ml of the growth medium. Growth was monitored by measuring culture turbidity at 540 nm. To reduce experimental errors,

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culture samples with turbidities higher than 0.5 were diluted with water.

The oxygen consumption rate of cells was measured in 10 mM phosphate buffer (pH 5.5) on an LP-7 polarograph (Czech Republic) equipped with a Clark-type oxygen electrode kept at 20–22°C. The final volume of the respiration medium was 2 ml. Cells were added in amounts of 0.5 to 1 mg dry wt. The concentration of dissolved oxygen in the respiration medium was taken to be 250 μM.

Experiments with the washed cells were carried out as follows. Logarithmic-phase cells, whose respiration was 95% sensitive to 1 mM cyanide, were washed twice with distilled water and suspended in a 10 mM phosphate buffer (pH 5.0) to a cell density of 3–3.5 mg dry wt/ml. The cell suspension prepared in this manner was incubated in a 750-ml flask on the shaker under conditions typical for growth experiments (200 rpm; 29°C).

cAMP was extracted from the yeast cells with 5% perchloric acid. To accomplish this, 4.5 ml of the cell suspension taken from the flask was introduced into a test tube containing 0.5 ml of 50% perchloric acid, thoroughly mixed, and incubated in an ice bath. The cAMP-containing acid extract was neutralized with 5 N KOH by vigorously stirring the mixture. The precipitate was removed by centrifugation or filtration through a paper filter. The filtrate was stored at –15°C until needed. cAMP was assayed by the conventional method using the kit purchased from Amersham [9].

RESULTS

It is known that the respiration of glucose-grown yeast cells becomes resistant to cyanide during their transition from the exponential to the stationary growth phase. When grown on ethanol, pyruvate, citrate, or acetate, the yeast cells exhibit cyanide-resistant respiration already in the early exponential phase [1].

In our experiments, the cyanide-resistant respiration of glucose-grown exponential phase yeast cells was induced by incubating them in a medium containing cyanide or antimycin A with or without a nonfermentable substrate (ethanol or acetate). As can be seen from Fig. 1, the respiration of the exponential-phase cells grown on glucose was 95% sensitive to 1 mM cyanide. The aerobic incubation of cells in the presence of cyanide (Fig. 1a, curve *1*) or antimycin A (Fig. 1a, curve *2*) without any substrate added led to the development of cyanide-resistant respiration sensitive to 5 mM BHA (Fig. 1a, curve *3*), thus indicating the induction of alternative oxidase. Cyanide-resistant respiration began to develop 30 min after the addition of the inhibitors and was maximum 3 h later.

In the presence of 0.5 M ethanol, the respiration of cells measured in the absence of cyanide first decreased (Fig. 1b, curve *1*) and then increased to the initial value after 60 min. Cyanide-resistant respiration began to develop after the 30-min incubation period, and was maximum 3 h later (Fig. 1b, curve *2*). These data can be interpreted to indicate the transition from glucose metabolism to the oxidation of ethanol. Similar results were obtained for the yeast cells that were incubated in the presence of acetate (Fig. 1c) or citrate (data not shown).

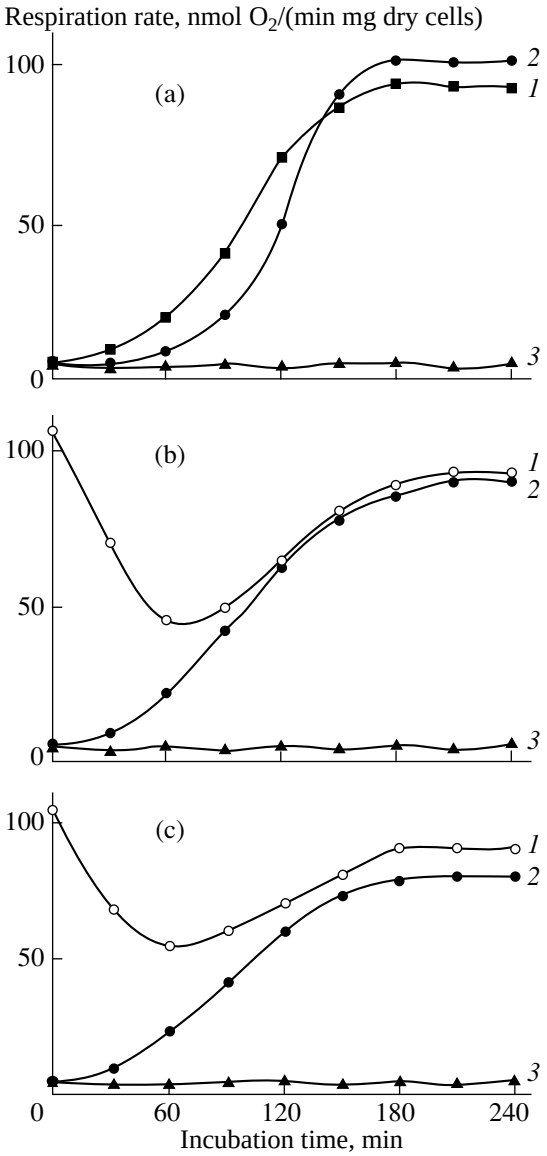

Fig. 1. Effect of cyanide, antimycin A, BHA, ethanol, and acetate on the cyanide-resistant respiration of *Y. lipolytica* cells. Panel a: cells incubated aerobically without any substrate added in the presence of 1 mM cyanide (curve *1*), 10 μM antimycin A (curve *2*), and a mixture of 1 mM cyanide and 5 mM BHA (curve *3*). Panel b: cells incubated aerobically in the presence of 0.5 M ethanol without any respiratory inhibitor added (curve *1*) or in the presence of 1 mM cyanide (curve *2*) and a mixture of 1 mM cyanide and 5 mM BHA (curve *3*). Panel c: cells incubated aerobically in the presence of acetate without any respiratory inhibitor added (curve *1*) or in the presence of 1 mM cyanide (curve *2*) and a mixture of 1 mM cyanide and 5 mM BHA (curve *3*).

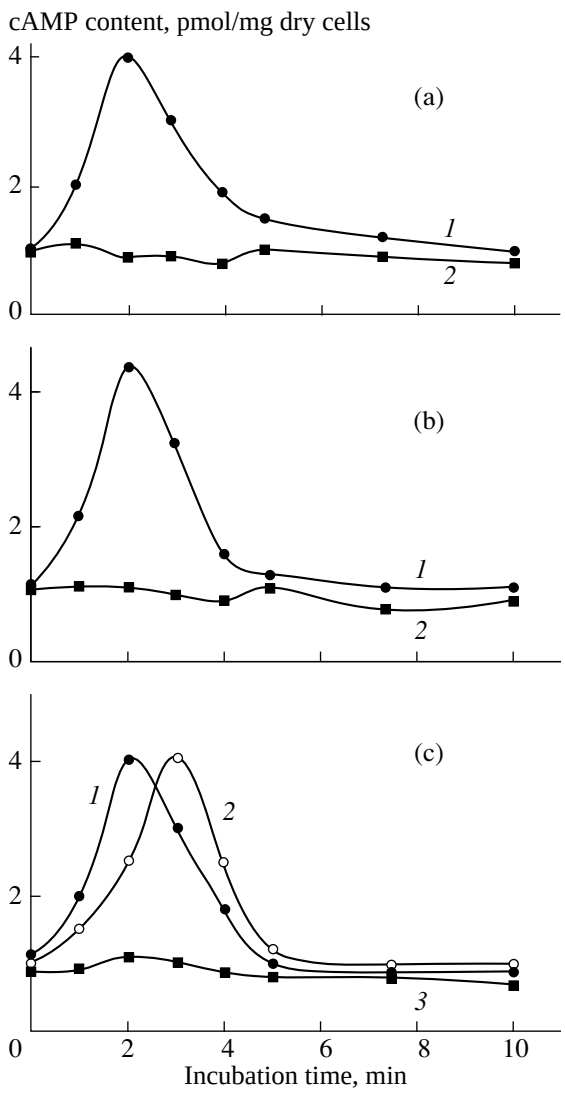

Fig. 2. Effect of (a) cyanide, (b) acetate, and (c) ethanol on the cAMP content of *Y. lipolytica* cells incubated under aerobic conditions. Panel a: (*1*) 1 mM cyanide; (*2*) without cyanide. Panel b: (*1*) 0.25 M acetate; (*2*) without acetate. Panel c: (*1*) 0.5 M ethanol; (*2*) 1 M ethanol; and (*3*) without ethanol.

Measurements of the cAMP content of cells incubated in the presence of cyanide, ethanol, or acetate showed that in all cases the cAMP content attained its maximum after 2 min of incubation (Fig. 2a, curve *1*) and then (within 5–6 min of incubation) decreased to the initial value. In the next 3 h of incubation, the cAMP content of cells did not change (data not shown).

Similar results were obtained for the cells incubated in the presence of acetate (Fig. 2b) or ethanol (Fig. 2c). In the latter case, the dynamics of the intracellular cAMP pool depended on the concentration of the ethanol added. For instance, when the cells were incubated in the presence of 0.5 and 1 M ethanol, their cAMP contents attained maxima after three and two minutes of incubation, respectively (Fig. 2c, curves *2* and *3*).

DISCUSSION

The alternative oxidase of fungi, yeasts, and plants can be induced by oxidative stress [6], heat shock [7], respiratory inhibitors [1, 8], nonfermentable substrates (organic acids and ethanol), the exhaustion of glucose in the growth medium, and so on [1, 4, 5]. This suggests that the induction of alternative oxidase is a component of the complex adaptive response of cells to stress factors.

The diversity of factors leading to the induction of alternative oxidase poses the problem of the inducing signal. The knowledge that antimycin A induces alternative oxidase and enhances the production of superoxide radicals in the respiratory chain of yeast allowed Minagava *et al.* to infer that alternative oxidase may be induced by active oxygen species [6]. In our opinion, however, this suggestion cannot explain the diversity of factors capable of inducing alternative oxidase.

The results presented in this paper show that cyanide, antimycin A, ethanol, and acetate induce the synthesis of alternative oxidase (Fig. 1) and raise the cAMP content of yeast cells (Fig. 2). These data, together with the finding that the alternative oxidase gene of *N. crassa* contains a CRE [3], suggest that it is cAMP that controls the expression of the alternative oxidase gene in yeasts.

It should be noted that a diversity of stress factors, such as heat shock, ethanol [9], organic acids [11], and membrane-depolarizing agents (cyanide and antimycin A) [10–15], can enhance the intracellular cAMP pool and induce alternative oxidase synthesis. The increased cAMP pool acts to decrease the cytoplasmic pH [11, 12, 16] and transmembrane potential [10, 14, 15]. In turn, low cytoplasmic pH values activate adenylate cyclase and suppress phosphodiesterase [12].

The mechanism of induction of alternative oxidase remains unknown. The experimental studies of the transcription of the stress protein genes of *Saccharomyces cerevisiae* showed the existence of a common stress response element (STRE) that controls the adaptive response of yeast cells to various stresses [17]. For instance, the transcription of the cytoplasmic catalase T gene (*CTT1*) is activated by various stresses, such as oxidative, osmotic, heat shock, and transition to the stationary growth phase [18], and is regulated by cAMP-dependent or -independent mechanisms [19, 20]. Taking into account that the conditions that induce the synthesis of catalase T and alternative oxidase are similar, it can be suggested that the transcription of the alternative oxidase gene is activated by similar mechanisms.

REFERENCES

1. Medentsev, A.G., Arinbasarova, A.Yu., and Akimenko, V.K., Regulation and Physiological Role of Cyanide-Resistant Oxidase in Fungi and Plants, *Biokhimiya*, 1999, vol. 64, no. 11, pp. 17–32.

2. Akimenko, V.K., Golovchenko, N.P., and Medentsev, A.G., The Absence of Energy Conservation Coupled with Electron Transfer via the Alternative Pathway in Cyanide-Resistant Yeast Mitochondria, *Biochim. Biophys. Acta*, 1979, vol. 545, no. 2, pp. 398–403.
3. Li, Q., Ritzel, R.G., McLean, L.L.T., McIntosh, L., Ko, T., Bertrand, H., and Nargang, F.E., Cloning and Analysis of the Alternative Oxidase Gene of *Neurospora crassa*, *Genetics*, 1996, vol. 142, no. 1, pp. 129–140.
4. Kapulnik, Y., Yalpani, N., and Raskin, I., Salicylic Acid Induced Cyanide-Resistant Respiration in Tobacco Cell-Suspension Cultures, *Plant Physiol.*, 1992, vol. 100, pp. 921–1926.
5. Vanlerberghe, G.C. and McIntosh, L., Signal Regulating the Expression of the Nuclear Gene Encoding Alternative Oxidase of Plant Mitochondria, *Plant Physiol.*, 1996, vol. 111, pp. 598–595.
6. Minagava, N., Koga, S., Nakano, M., Sakajo, S., and Yashimoto, A., Possible Involvement of Superoxide Anion in the Induction of Cyanide-Resistant Respiration in *Hansenula anomala*, *FEBS Lett.*, 1992, vol. 302, pp. 317–219.
7. Habel, D., Plesofsky-Vig, N., and Brambl, R., The Respiratory Response to Heat Shock in *Neurospora crassa*, *FEMS Microbiol. Lett.*, 1991, vol. 81, pp. 317– 322.
8. Vanderleyden, J., Hanssens, L., and Verachtert, H., Induction of Cyanide-Insensitive Respiration in *Moniliella tomentosa* by the Use of *n*-Propanol, *J. Gen. Microbiol.*, 1978, vol. 105, pp. 63–68.
9. Kallies, A., Gebauer, G., and Rensing, L., Heat Shock Effects on Secondary Messenger Systems of *Neurospora crassa*, *Arch. Microbiol.*, 1998, vol. 170, pp. 191–200.
10. Trevillyan, J.M. and Pall, M.L., Control of Cyclic Adenosine 3',5'-Monophosphate Levels by Depolarizing Agents in Fungi, *J. Bacteriol.*, 1979, vol. 2, pp. 397–403.
11. Thevelein, J.M., Beullens, M., Honshoven, F., Hoebeack, G., Detremerie, K., Hollander, I.A.D., and Jans, A.W.H., Regulation of the cAMP Level in the Yeast *Saccharomyces cerevisiae*: Intracellular pH and the Effect of Membrane Depolarizing Compounds, *J. Gen. Microbiol.*, 1987, vol. 133, no. 8, pp. 2191– 2196.
12. Thevelein, J.M., Signal Transduction in Yeast, *Yeast*, 1994, vol. 10, no. 13, pp. 1753–1790.
13. Craig, E.A. and Gross, C.A., Is *hsp 70* the Cellular Thermometer?, *Trends Biochem. Sci.*, 1991, vol. 16, pp. 135–140.
14. Pall, M.L., Cyclic AMP and the Plasma Membrane Potential in *Neurospora crassa*, *J. Biol. Chem.*, 1977, vol. 252, pp. 7146–7150.
15. Pall, M.L., Adenosine 3',5'-Phosphate in Fungi, *Microbiol. Rev.*, 1981, vol. 45, pp. 462–480.
16. Sanders, D., Hansen, U.P., and Slayman, C.L., Role of the Plasma Membrane Pump in pH Regulation in Non-Animal Cells, *Proc. Natl. Acad. Sci. USA*, 1978, vol. 78, pp. 5903–5907.
17. Ruis, H. and Schuller, C., Stress Signalling in Yeast, *BioEssays*, 1995, vol. 17, no. 11, pp. 959–965.
18. Marchler, G., Schüller, C., Adam, G., and Ruis, H.A., *Saccharomyces cerevisiae* UAS Element Controlled by Protein Kinase A Activates Transcription in Response to a Variety of Stress Conditions, *EMBO J.*, 1993, vol. 12, no. 5, pp. 1997–2003.
19. Bissinger, P.H., Weiser, R., Schanz, M., Adam, G., Hartig, A., and Ruis, H., Control of *Saccharomyces cerevisiae* Catalase T Gene (*CTT1*) Expression by Nutrient Supply via the RAS–Cyclic AMP Pathway, *Mol. Cell Biol.*, 1989, vol. 9, pp. 1309–1315.
20. Belazzi, T., Wagner, A., Weiser, R., Schanz, M., Adam, G., Hartig, A., and Ruis, H., Negative Regulation of Transcription of the *Saccharomyces cerevisiae* Catalase T (*CTT1*) Gene by cAMP Is Mediated by a Positive Control Element, *EMBO J.*, 1991, vol. 10, no. 3, pp. 585–592.