A Spontaneous Change in the Intracellular Cyclic AMP Level in *Aspergillus niger* Is Influenced by the Sucrose Concentration in the Medium and by Light

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Received 13 December 1996/Accepted 21 April 1997

A spontaneous rise in intracellular cyclic AMP (cAMP) levels was observed in the early stages of *Aspergillus niger* growth under conditions yielding large amounts of citric acid. The amount of cAMP formed was found to depend on the initial concentration of sucrose in the medium. Under higher-sucrose conditions, the cAMP peak appeared earlier and was higher, while in lower-sucrose media a flattened peak was observed later in fermentation. Since in media with higher concentrations of sucrose intracellular citric acid starts to accumulate earlier and more rapidly, cAMP synthesis may be triggered by intracellular acidification, which is caused by the dissociation of citric acid. No spontaneous increase in cAMP concentrations could be detected when the cells were grown in continuously illuminated cultures, suggesting that *A. niger* phosphodiesterase (PDE) is photoregulated. More evidence for the activation of PDE by light was obtained from morphological studies under light and dark conditions in the presence of cAMP or N₆,O₂'-dibutyryl cAMP, and this idea was additionally supported by experiments in which PDE inhibitors were tested.

In many organisms, cyclic AMP (cAMP) has been found to control a wide variety of often apparently unrelated functions, most commonly in a second messenger role. The most-intensive studies were originally made with higher-order animals and with *Escherichia coli*, but evidence has recently been obtained which links cAMP to the control of a number of functions in fungi as well, including utilization of endogenous and exogenous carbon sources (in *Saccharomyces* species), conidiation (in *Neurospora crassa*), dimorphism (several fungi), and the sexual process and phototropism (in *Phycomyces* species) (16).

There have also been several reports of cAMP levels in *Aspergillus niger*, mostly under citric acid-accumulating conditions; however, the results seem to be conflicting. Al Obaidy and Berry have postulated that high levels of cAMP are principally associated with an optimum physiological state for citric acid production (1), while Xu et al. claim that cAMP levels are not influenced by manganese deficiency and do not correlate with citric acid accumulation (26).

In our lab, a spontaneous increase in cAMP levels during the early stage of *A. niger* growth under citric acid-accumulating conditions was observed (10), as was phosphorylation of 6-phosphofructo-1-kinase by cAMP-dependent protein kinase (9), which apparently activated the enzyme and increased the glycolytic flux at the expense of the pentose phosphate pathway. Simultaneously with the cAMP peak, a change in morphology from enlarged bulbous cells to filamentous hyphae took place (8) and citric acid started to be excreted into the medium (12). These preliminary results suggest that changes in cAMP concentration are important in regulating metabolism.

To elucidate the role of cAMP in *A. niger*, the amounts of intracellular cAMP under different environmental conditions were monitored.

MATERIALS AND METHODS

A. niger (MZKI A60) spores were harvested from 7-day-old wort agar slants and were suspended in 25 ml of sterile 0.1% Tween 80 solution. A suspension of spores was used to inoculate the chemically defined medium as described previously (8). However, with different initial sucrose concentrations, the amounts of inorganic salts remained unchanged. Sterilization was achieved through pasteurization, which proved to be sufficient due to the low pH (2.5) of the medium.

For morphological observations, fermentations were run on a rotary shaker (model G-52; New Brunswick Scientific Co., Inc., Edison, N.J.) in 500-ml flasks with baffles containing 100 ml of substrate. The flasks were shaken at 100 rpm and 30°C. Mycelia were observed under a microscope (Wild, Heerbrugg, Switzerland).

For determinations of biomass (dry weight), intracellular citric acid levels, and cAMP levels, the fermentations were run in a glass stir tank bioreactor (Bio-engineering, Wald, Switzerland) with a 5-liter working volume. Unless otherwise stated, 5 liters of medium was inoculated with 10⁹ spores. The temperature was kept at 30°C, and the medium was aerated with 5 liters of air/min. To keep the culture under dark conditions, the glass wall of the fermentor was wrapped with black paper.

The amount of biomass was determined gravimetrically after the cells were dried at 105°C to a constant weight.

To detect intracellular levels of citric acid, the mycelia were washed with an ice-cold solution of 31 mM HCl (pH = 1.5). Then, the mycelia (about 0.1 g [dry weight]) were homogenized with a glass bead disintegrator (Braun, Melsungen, Germany) and extracted with 0.6 M perchloric acid. The homogenate was subsequently centrifuged at 1,000 × g for 10 min, and the supernatant was neutralized with 2 N KOH. After filtration through a Whatman GF/C filter, the amount of citric acid in the filtrate was enzymatically determined with the Boehringer (Mannheim, Germany) kit.

For cAMP determinations, approximately 100 mg (wet weight) of mycelia was collected from the fermentation broth by filtration through a Whatman GF/C glass microfiber filter, rinsed with an ice-cold 1 mM EDTA solution, and immediately frozen under liquid nitrogen. The sampling procedure was terminated in less than 30 s. After being weighed, the samples were homogenized in a glass bead disintegrator (Braun), and cAMP was extracted with perchloric acid and prepared for evaluation as described previously (23). Finally, cAMP levels were measured with a cAMP test kit (Amersham International, Amersham, Buckinghamshire, United Kingdom).

When the influence of light was studied, flasks on a shaker or glass fermentor were constantly illuminated with a daylight fluorescence lamp (Biolum L36W/72, 6,500 K and 2,300 lm; Osram, Germany) from a distance of approximately 30 cm.

All data presented are the averages of results obtained from three or more independent measurements.

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FIG. 1. Time profiles of cAMP levels in mycelia grown in substrates with different initial sucrose concentrations (25% [■], 20% [▲], 15% [●], 10% [✕], and 5% [▼]).

RESULTS

cAMP levels. Changes in cAMP levels detected in mycelia grown in media with different initial concentrations of sucrose are shown in Fig. 1. It can be seen that with higher amounts of sucrose in the medium, the peak of the intracellular cAMP concentrations appeared earlier and reached higher values, while with lower levels of sucrose, the increase in cAMP levels was not so obvious and took place later in fermentation. During growth on 1% sucrose, virtually no increase in cAMP levels could be detected. The increase in cAMP concentrations was transient, and, after reaching maximal values, it decreased. Again, with higher concentrations of sucrose this effect seemed to be more rapid, and initial cAMP values were restored after 6 to 8 h, while under conditions of lower sucrose concentrations, the subsequent decrease appeared to be particularly slower.

Effect of light. However, a spontaneous increase in cAMP levels could be observed only in the cultures grown under dark conditions. When the fermentation broth in a glass bioreactor was continuously illuminated by a daylight fluorescence lamp, the intracellular amount of cAMP remained low. However, in the presence of 5 mM 3-isobutyl-1-methylxanthine (IBMX), an inhibitor of phosphodiesterase (PDE), a slight increase in

FIG. 2. Intracellular cAMP levels of constantly illuminated cultures grown in 15% sucrose medium with (■) or without (●) 5 mM IBMX.

FIG. 3. The amounts of citric acid (in millimoles per liter of cell volume) as detected at 24 h of fermentation in cells grown in substrates with different initial sucrose concentrations.

cAMP levels was detected in spite of the light conditions (Fig. 2).

Intracellular citric acid. The amounts of citrate in the cells at 24 h of fermentation, when no extracellular citrate could yet be detected, were determined. With different initial sucrose concentrations, higher levels were found in media with more sucrose and vice versa (Fig. 3). In media with higher percentages of sucrose, intracellular citrate started to accumulate earlier and more rapidly than in cells growing at lower concentrations (data not shown).

Growth rate. By monitoring the increase in biomass (dry weight) in substrates with different sucrose concentrations, the highest growth rate was observed with the highest carbohydrate concentration, and the growth rate decreased concomitantly as the amount of sucrose in the medium was decreased (Fig. 4).

Amount of inoculum. When the levels of cAMP in 15% sucrose medium that was inoculated with different amounts of spores were measured, higher peak cAMP levels were detected in mycelia developed from denser inocula. At densities of approximately 10^7 spores per 5 liters of substrate, barely ob-

FIG. 4. Time profiles of the increase in biomass (dry weight) in mycelia with different initial sucrose concentrations (25% [■], 20% [▲], 15% [●], 10% [✕], and 5% [▼]).

FIG. 5. Influence of the amount of inoculum on cAMP levels in mycelia in 15% sucrose medium. The inocula contained 10^9 (■), 10^8 (▲), and 10^7 (●) spores.

servable peaks could be detected, while with 10^9 spores, the maximal concentration of cAMP was approximately fourfold higher (Fig. 5).

Hyperosmotic shock. As reported previously, hypoosmotic shock resulted in rapid synthesis of cAMP (10); however, no

such effect could be detected after a sudden increase in sucrose concentration from 15 to 30%.

Morphology of germination. The enzyme responsible for breakdown of cAMP is cAMP-specific PDE, which has been well studied in higher eukaryotes (2). Since some isoenzymes of the PDE family are known to be activated by light (6), the effect of constant illumination on *A. niger* cells was studied. It was not possible to detect any cAMP peak in mycelia grown under light conditions, and no citric acid started to accumulate in the fermentation broth. By monitoring the morphology of the fungus, only enlarged bulbous cells could be observed under light conditions, and there was no spontaneous change to filamentous growth. As reported previously, after 24 to 26 h of growth in the dark, small buds appeared on the surfaces of the bulbous cells from which filamentous hyphae later developed (8). Supplementation of culture medium with 5 mM cAMP resulted in the development of filamentous hyphae directly from the spores only under dark conditions, while in illuminated cultures enlarged bulbous cells were formed. However, by germinating the spores in the presence of 5 mM N_6, O_2' -dibutyryl cAMP (dbcAMP), a lipid-soluble derivative of cAMP, normal filamentous growth appeared in spite of constant radiation by visible light (Fig. 6).

Morphology could also be affected by the inhibitors of PDE. The germination of spores under light conditions in the presence of 5 mM cAMP and 10 mM inhibitor, such as IBMX, Ca^{2+} , dipyrindamole, and amrinone, was in a form of filamen-

FIG. 6. Morphology of 18-h-old *A. niger* cells in 15% sucrose medium supplemented with 5 mM cAMP (a and b) or 5 mM dbcAMP (c and d) under dark (a and c) and light (b and d) conditions. Magnification, $\times 500$.

FIG. 7. Morphology of 18-h-old *A. niger* cells in 15% sucrose medium supplemented with 5 mM cAMP under light conditions and in the presence of the following specific PDE inhibitors (each at 10 mM): IBMX (a), CaCO_3 (b), amrinone (c), dipyrindamole (d), papaverine (e), and theophylline (f). Magnification in panels a, b, c, and d in panels e and f, $\times 500$ and $\times 750$, respectively.

tous hyphae. However, papaverine and theophylline in a 10 mM concentration and with 5 mM cAMP enabled the formation of enlarged bulbous cells when the cultures were kept constantly illuminated (Fig. 7).

DISCUSSION

Before now, the role of cAMP as a signal-transducing molecule in fungi has been reviewed by several authors (5, 16, 22).

This important intracellular regulator has also been studied for *A. niger*, yet mostly in regard to citric acid fermentation. In 1973, Wold and Suzuki (25) observed an increased rate of citric acid synthesis after addition of cAMP to the medium. However, in their experiments, a medium yielding a low amount of citric acid with less than 1% of the initial sucrose concentration was used, which did not allow a high rate of citric acid synthesis. More interesting results were described by Al Obaidi and Berry (1), who followed the changes in intracellular cAMP

during fermentation yielding a high amount of citric acid. They concluded that high cAMP levels were principally associated with an optimum physiological state for citric acid production. In 15% sucrose medium and with an initial pH of 2.9, a peak in the cAMP concentration at about 24 h was observed, which is in accordance with our findings. From their results, it could be clearly seen that higher citric acid yields were obtained with mycelia initially exhibiting higher cAMP peaks.

cAMP levels in *A. niger* were also monitored by Xu et al. (26). Their data concerning cAMP levels in mycelia grown under low-sucrose-concentration conditions are in accordance with our findings, according to which a flattened peak was observed later in fermentation. However, under citric acid-producing conditions (with 14% sucrose in the medium), they were unable to detect any significant changes in cAMP levels. Since the samples were taken only at 24-h intervals, it may be that a peak in cAMP concentration was simply missed and a wrong conclusion was made, i.e., that the changes in cAMP levels do not correlate with citric acid accumulation. In our case, in 15% sucrose medium an increase in cAMP concentration started at approximately 25 h, and the concentration reached its maximum value after 3 to 4 h and decreased to its original level in the ensuing 10 h. It is logical for a molecule functioning as an intracellular mediator to be tightly controlled, and, after synthesis, it should be rapidly degraded to respond to another signal after a time.

A spontaneous increase in cAMP concentration could be caused by intracellular acidification. A low intracellular pH is known to be a potent stimulator of the RAS-adenylate cyclase signaling pathway in a number of fungal species, including *Saccharomyces cerevisiae* (21, 23, 24), *N. crassa* (17, 24), and *Mucor racemosus* (24). In *A. niger*, intracellular acidification was most probably caused by citric acid, which accumulated in the cells during the early stages of growth in a medium yielding high levels of citric acid. Citric acid with a pK' of 3.13 dissociated at physiological pH values and released protons, which consequently decreased the pH (11).

A rapid increase in cAMP levels could also be triggered by the addition of azide to the medium (data not shown), which is a substance known to cause a decrease in intracellular pH (23); this additionally supported the hypothesis of spontaneous stimulation of endogenous cAMP synthesis by intracellular acidification.

The enzyme which mediated the cAMP signal to target proteins was cAMP-dependent protein kinase. It was found to phosphorylate 6-phosphofructo-1-kinase, which is one of the key regulatory enzymes of glycolysis (9). After phosphorylation, the enzyme was significantly activated, which might contribute to higher metabolic flow through glycolysis. Undisturbed glycolytic flux was shown to be prevalent during citric acid accumulation (13); on the other hand, it seemed to stimulate growth as well: namely, in mycelia with higher initial cAMP peaks, more rapid growth was observed.

It is important to realize that no changes in cAMP concentration could be detected when the culture was constantly illuminated. Since some increase was detected in the presence of IBMX, an inhibitor of PDE, it could be that this enzyme was activated by light, as reported for some other organisms. Light was reported to stimulate cGMP hydrolysis by cGMP-dependent PDE in vertebrate photoreceptors (6), while among microorganisms, photoregulation of cAMP-specific PDE activity was described for *N. crassa* (19) and in *Phycomyces blakesleeanus* (4). Therefore, it seemed very likely that in *A. niger* grown under light conditions, a highly active enzyme hydrolyzed cAMP more rapidly than adenylate cyclase could synthesize it.

The addition of cAMP had an effect on cell morphology

under only dark conditions, while dbcAMP stimulated filamentous growth in illuminated substrates as well. It should be noted that fungal membranes were relatively impermeable to cAMP molecules (5) and that in spite of high extracellular concentrations the amounts of molecules in the cells were relatively low. They could not be degraded by low active PDE under dark conditions, while light-activated enzyme could reduce the amount of cAMP to a level which was unable to further influence fungal differentiation. On the other hand, a more-lipophilic cAMP analog, i.e., dbcAMP, was believed to enter the cells easily, so that even light-activated PDE could not break down all the molecules.

The experiments in which the influence of light on cell morphology was studied supported the hypothesis of light-activated PDE in *A. niger* and most probably showed the involvement of cAMP in cell wall synthesis, which was reflected in the shape of the cells (20). The possible role of cAMP in development and morphogenesis has especially attracted the attention of people working with dimorphic fungi. In *Histoplasma capsulatum*, cAMP levels were found to be higher in mycelia than in yeast cells and dbcAMP converted yeast cells to hyphal growth (14). A similar effect of changes in cAMP levels on morphology was described for *Candida albicans* (3). The pattern in *A. niger* appeared to resemble that found in *Histoplasma* and *Candida* species, in which low cAMP levels were associated with bulbous growth while filamentous hyphae developed after a peak in cAMP levels. However, the relationship between cAMP and morphology might be different in *Mucor* sp., in which cAMP was postulated to produce yeast-like growth (15). Moreover, in *M. racemosus* and *Mucor rouxii*, exogenous dbcAMP induced a transformation from the mycelial to a yeast-like form (7, 18).

For mammalian tissues, multiple isoenzymes of cyclic nucleotide PDEs which could be selectively inhibited by specific inhibitors have been described (2). In order to learn more about the *A. niger* enzyme, the influence of different inhibitors on morphology was monitored. However, from the data obtained, it was impossible to conclude to which group of PDEs the *A. niger* enzyme belongs, and a final decision must await full characterization of the cloned gene and/or purified enzyme.

ACKNOWLEDGMENT

This work was supported by the Ministry of Science and Technology, Ljubljana, Slovenia (grant J1-5018-104).

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