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Control of β-Glucosidase Synthesis in *Mucor racemosus*

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The β-glucosidase of *Mucor racemosus* was shown to be synthesized when the organism was grown in the presence of such diverse carbon sources as glycerol, lactate, xylose, ribose, α-methylglucoside, α-phenylglucoside, maltose, and cellobiose. Enzyme synthesis was strongly repressed in the presence of hexoses. In addition, exogenous cyclic adenosine 3',5'-monophosphate (cAMP) resulted in enzyme repression. When cAMP was added exogenously after enzyme activity had accumulated, a reversible enzyme inactivation occurred. Growth on disaccharides (maltose or cellobiose) was severely retarded in the presence of cAMP, whereas that on glucose remained unaffected. The results indicate a probable role for cAMP in control of glucosidase synthesis in *Mucor*.

Mucor racemosus is a dimorphic fungus which grows in a mycelial form aerobically and as spherical, budding yeast cells when grown in a carbon dioxide atmosphere. Larsen and Sypherd (11) and Paznokas and Sypherd (16) have shown that intracellular cyclic adenosine 3',5'-monophosphate (cAMP) plays a key role in the control of morphology in *M. racemosus*. Yeast cells grown in CO_2 contain elevated cAMP levels, which drop four- to fivefold when the cells are shifted to aerobic conditions. Mycelia develop subsequent to the alteration of cAMP levels. If exogenous N^6,O^2-dibutyryl cAMP is added at the time of shift, the morphology remains yeastlike. In addition, when N^6,O^2-dibutyryl cAMP is added to an aerobically growing mycelial culture, a hyphae-to-yeast transition occurs.

Yeast development, whether in a carbon dioxide atmosphere or in air plus N^6,O^2-dibutyryl cAMP, requires the presence of a hexose carbon source. In contrast, mycelial cells can use a wide variety of carbon sources, including disaccharides and pentoses (2). Additionally, experiments performed in this laboratory show that yeastlike growth requires the presence of an organic nitrogen source (J. Peters, unpublished observations). Thus, the yeast cells develop during conditions that resemble extreme catabolite repression.

The catabolite repression of β-galactosidase in enteric bacteria is mediated through depletion of intracellular cAMP levels when the bacteria are grown on a repressing carbon source such as glucose (13, 17, 19). We have investigated the role cAMP might play in the observed effects of glucose on the physiology of *M. racemosus*. Our experiments show that the β-glucosidase of *M. racemosus* is regulated in a unique fashion. Unlike most other microbial glycosidases, the enzyme does not require an exogenous inducer but is subject to hexose repression. Our experiments also show that cAMP does not relieve the repression by hexoses but may play a negative role in the synthesis of the enzyme. Furthermore, we show that the enzyme is inactivated in cells treated with cAMP.

MATERIALS AND METHODS

Organism. *M. racemosus* ATCC 1216B was used in all experiments. Sporangiospore inocula were prepared by washing the surface of yeast extract-peptone-glucose agar plates (11) with sterile distilled water. The spores were harvested by centrifugation, washed twice with sterile distilled water, and suspended in 20% glycerol at the desired density. Spores could be stored frozen in glycerol for at least 30 days without loss of viability.

Media and culture conditions. A defined medium, devised by J. Peters in this laboratory, was used in all experiments. The medium contained, per liter: carbon source, 10 g; NH_4NO_3, 1.5 g; sodium glutamate, 1.5 g; yeast nitrogen base (Difco), 0.17 g. All medium components were adjusted to pH 4.5 with H_2SO_4 and autoclaved separately. Cultures were inoculated to 10^6 sporangiospores per ml and were incubated at 22°C on a shaker rotating at 200 rpm.

Enzyme assays. Glucosidase was assayed by rendering the cells permeable with dimethyl sulfoxide (1). One-milliliter culture samples were removed and added to 1 ml of dimethyl sulfoxide. The cells were incubated at 22°C for 15 min; after centrifugation, the solvent was removed and the cells were

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washed twice with 5 ml of distilled water. The cell pellet was suspended in 100 μl of 50 mM sodium acetate buffer, pH 4.5. The enzyme reaction was begun by the addition of 100 μl of 10 mM p-nitrophenyl-β-D-glucoside. Enzyme reactions were performed at 40°C and were proportional to protein concentration and linear with time. The reactions were stopped by the addition of 0.8 ml of 0.2 M Na_2CO_3. Cells were removed by centrifugation, and the absorbance of the supernatant at 400 nm was read in a Zeiss PMQ II spectrophotometer. One unit of enzyme activity was defined as that amount of enzyme hydrolyzing 1 nmol of pNPG per min.

Protein determination. Cell protein was determined by heating cell pellets in 1 N NaOH for 20 min in a boiling-water bath. Insoluble debris was removed by centrifugation, and soluble protein in the supernatant was estimated by the method of Lowry et al. (12).

Chemicals. cAMP, cycloheximide, actinomycin D, p-nitrophenyl-α-D-glucose, and dimethyl sulfoxide were obtained from Sigma Chemical Co. Netropsin was a gift from Calvin McLaughlin.

RESULTS

Effect of carbon source on β-glucosidase synthesis. *Mucor* was grown in defined media with various carbon sources present at a concentration of 1% (wt/vol). During logarithmic growth, the specific activities of β-glucosidase were determined at intervals. The maximum specific activities derived on the specified carbon sources are presented in Table 1. High specific activities were obtained on pentoses (ribose and xylose), glycolytic intermediates (glycerol and lactate), disaccharides (maltose and cellobiose), and disaccharide analogues (α-methylglucoside and α-phenylglucoside). Growth on any of the hexose carbon sources led to a repression of enzyme synthesis. These results indicate that the presence of an exogenous inducer is not required for β-glucosidase synthesis and that enzyme synthesis may only respond to the catabolite repression. Similar results were obtained when α-glucosidase was assayed using p-nitrophenyl-α-D-glucoside as the substrate (data not presented). Both enzymes exhibit an apparent lack of induction and are synthesized only in the absence of a hexose.

Effect of inhibitors of RNA and protein synthesis on β-glucosidase synthesis. When cells grown on a glucose medium were shifted to a medium containing cellobiose, β-glucosidase activity appeared in about 1 h (Fig. 1). When cycloheximide or actinomycin D plus netropsin were added concomitant with the shift, enzyme accumulation was inhibited. These data are consistent with a requirement for RNA and protein synthesis.

Effect of glucose and cAMP on β-glucosidase synthesis. Figure 2 shows a differential plot of enzyme synthesis when *Mucor* is grown on the nonrepressing carbon source xylose. In the control culture, enzyme activity accumulated after spore germination and germ tube formation. When glucose (0.05%) or cAMP was added at a point in growth before high enzyme levels had appeared, the enzyme activity accumulated at a greatly reduced rate. Addition of glucose and cAMP together further reduced enzyme appearance. This experiment indicates that both glucose and cAMP act to prohibit further accumulation of glucosidase activity but cannot distinguish between cAMP acting to

TABLE 1. *Maximal specific activity of β-glucosidase from M. racemosus grown on various carbon sources*

Carbon source	Sp act (units/mg of protein)
Ribose	325
Xylose	354
Glycerol	430
Lactate	297
Maltose	247
Cellobiose	245
α-Methylglucoside	270
α-Phenylglucoside	259
Glucose, Fructose, Mannose, Galactose	<0.5

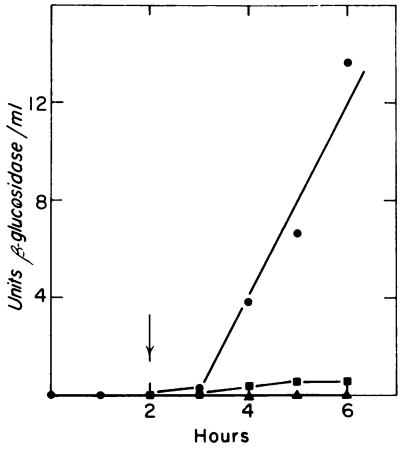

FIG. 1. *Inhibition of β-glucosidase synthesis in the presence of 200 μg of cycloheximide (▲) per ml and 100 μg of actinomycin D + 100 μg of netropsin (■) per ml. Cells were shifted from 1% glucose-containing medium to a 1% cellobiose-containing medium, and additions were made at the time indicated by the arrow.*

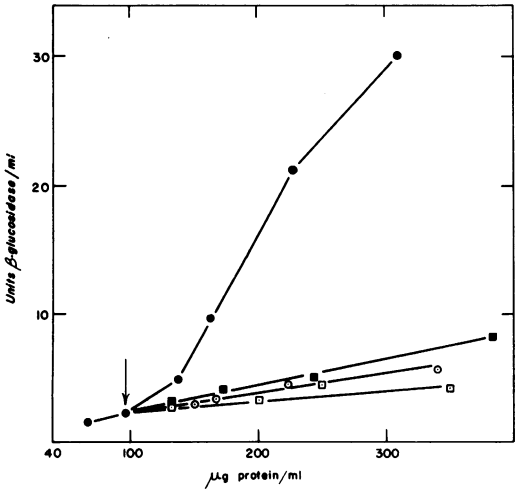

FIG. 2. *Effect of 7.5 mM cAMP* (■), *0.05% glucose* (○), *and 7.5 mM cAMP + 0.05% glucose* (□) *on the differential rate of β-glucosidase synthesis of cells growing on a 1% xylose-containing medium. Additions were made at the point in growth indicated by the arrow. No additions were made to the control culture* (●).

repress β-glucosidase synthesis and cAMP inactivating newly formed enzyme.

Inactivation of β-glucosidase in the presence of cAMP. Figure 3 shows the results of an experiment in which cAMP or glucose was added to a xylose-grown culture at two times after significant amounts of β-glucosidase had accumulated. At either time, addition of glucose resulted in preventing further enzyme accumulation, suggesting a repression of enzyme synthesis. cAMP addition caused a rapid loss of activity, indicating that at least an inactivation had occurred. No effect of 7.5 mM cAMP on enzyme activity in vitro using either dimethyl sulfoxide-treated cells or whole-cell extracts could be demonstrated.

The in vivo inactivation of β-glucosidase in the presence of cAMP was reversible (Fig. 4). In this experiment a culture growing on cellobiose and synthesizing β-glucosidase was divided, and cAMP was added to one portion. An inactivation occurred in the presence of cAMP, whereas enzyme accumulated in the control. After 2 h, the culture containing cAMP was again divided and cAMP was removed from one portion; then cycloheximide was added. The cells remaining in the presence of cAMP failed to regain enzyme activity, whereas the portion from which cAMP was removed regained activity in the absence of any new protein synthesis (i.e., in the presence of cycloheximide). The level of reactivated enzyme was approximately

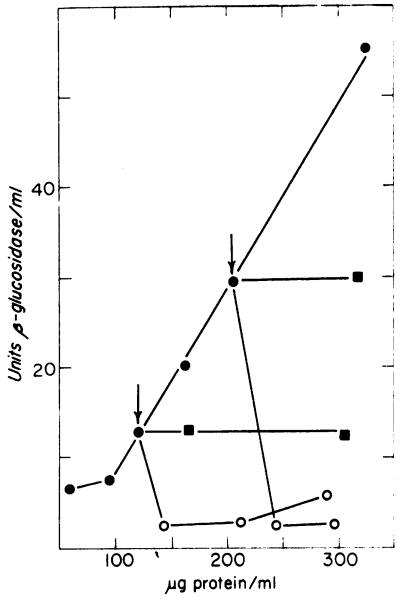

FIG. 3. *Effect of 7.5 mM cAMP* (○) *and 0.05% glucose* (■) *on the differential rate of β-glucosidase synthesis of cells grown on a 1% xylose-containing medium. Portions of the culture were divided from the control culture* (●) *at the two points indicated by the arrows and additions were made.*

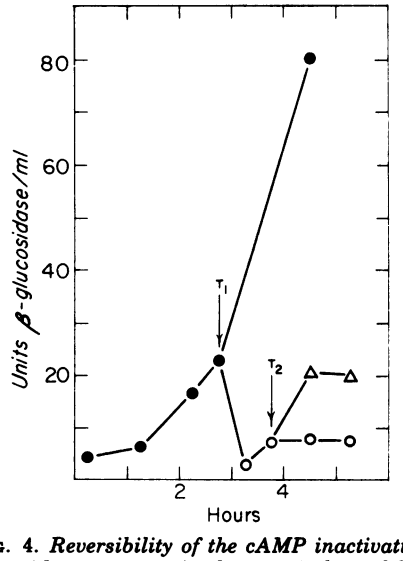

FIG. 4. *Reversibility of the cAMP inactivation of β-glucosidase activity. At the time indicated by the first arrow* (T_1) *a portion of the culture growing on a 1% cellobiose-containing medium was split from the control and 7.5 mM cAMP was added* (○). *The cAMP-containing culture was divided at the time indicated by the second arrow* (T_2), *a portion remained in the presence of cAMP* (○), *cAMP was removed from the second portion, and 200 μg of cycloheximide per ml was added* (△). *No additions were made to the control culture* (●).

equal to that found before the addition of cAMP. These results suggest that the cAMP not only inactivated β-glucosidase, but also repressed synthesis of new enzyme. The reappearance of enzyme after removal of cAMP indicated that the enzyme was not proteolytically degraded or excreted and suggested that a reversible enzyme modification occurred.

Effect of cAMP analogues on β-glucosidase levels. The results of experiments designed to determine whether cAMP analogues affect β-glucosidase are presented in Fig. 5. Neither adenosine 5'-monophosphate, adenosine, nor adenine influenced the differential rate of enzyme synthesis.

Effect of cAMP on growth. The inactivation and possible repression of β-glucosidase leads to a prediction that growth on cellobiose would be inhibited in the presence of cAMP. Figure 6 shows that this is the case. In this experiment a culture growing on glucose was divided, and two portions were allowed to remain in glucose medium ± cAMP. Another two portions were shifted to a cellobiose medium ± cAMP. In the glucose cultures, cAMP showed only a slight inhibition of growth rate compared to the control. The culture shifted to cellobiose in the absence of cAMP showed a diauxic lag followed

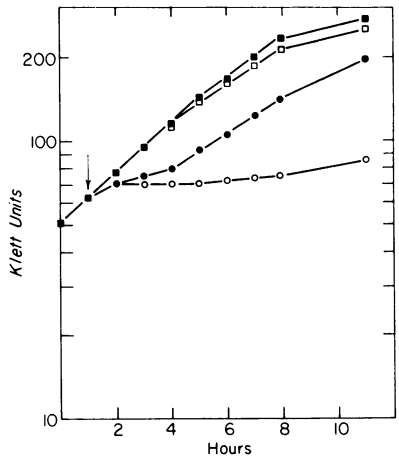

FIG. 6. *Effect of 7.5 mM cAMP on growth in glucose- or cellobiose-containing medium. A culture growing on a 1% glucose-containing medium was divided at the time indicated by the arrow. Two portions remained in the glucose medium, one in the presence of 7.5 mM cAMP* (□) *and the other in the absence of cAMP* (■). *Two additional portions were shifted to a 1% cellobiose-containing medium, one in the presence of 7.5 mM cAMP* (○) *and the other in the absence of cAMP* (●). NH_4NO_3 *was the sole nitrogen source used in this experiment.*

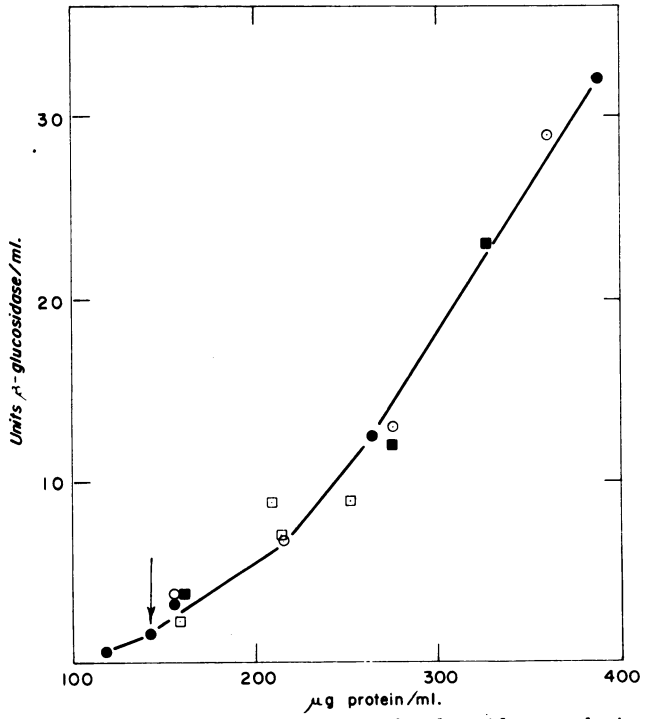

FIG. 5. *Effect of cAMP analogues on the differential rate of β-glucosidase synthesis of cells grown on a 1% xylose-containing medium. A culture was divided and additions were made at the point in growth indicated by the arrow. The additions were: 7.5 mM 5'-AMP* (○); *7.5 mM adenosine* (■); *7.5 mM adenine* (□); *no additions* (●).

by resumption of growth. The culture in cellobiose plus cAMP was severely growth retarded. This indicates that β-glucosidase inactivation was sufficient to effectively starve the cells for carbon.

DISCUSSION

A large body of literature exists concerning the control of synthesis of microbial glycosidases. In general, these enzymes are inducible by their substrates or substrate analogues and are repressed in the presence of preferred carbon sources such as glucose. In the fungi, the maltase (9) of *Saccharomyces* and the cellobiase (4) of *Neurospora* are controlled in this manner. In *Neurospora* another group of glycosidases, including invertase (14), trehalase (8), and an acid β-galactosidases (3), is induced by either disaccharides or certain monosaccharides. The α- and β-glucosidase from *Mucor* exhibit even less stringent control than this latter class of enzymes. Both are synthesized in the presence of any carbon source other than a hexose. The enzymes are depressed maximally on such diverse carbon sources as pentoses and glycolytic intermediates. Although the existence of an internal inducer cannot be disproven, it is tempting to postulate that the enzymes are not induced but are only subject to catabolite repression. Invertase in *Saccharomyces* is regulated in a similar fashion (15).

The relationship of intracellular cAMP levels to catabolite repression in the fungi is unclear at present. Van Wijk and Konijn (21) and Sy and Richter (18) have reported that as in bacteria, cAMP levels in *Saccharomyces* are elevated under nonrepressing conditions. In addition, exogenous cAMP has been shown to relieve glucose repression of respiratory development (5). Several other nucleotides, however, were as active in overcoming the repression of mitochondriogenesis (5). Additionally, in a mutant of *Saccharomyces* resistant to hexose repression of invertase synthesis no correlation with cAMP levels could be drawn (15). Recently it was demonstrated that in *Mucor* (16) and in *Aspergillus nidulans* (23) cAMP levels during growth reach their maximum while glucose concentrations are still high. It is interesting to note that cAMP levels in *Mucor* reach their maximum during sporangiospore germination (16) and that maximal β-glucosidase accumulation occurs subsequent to germination. It may be that the elevated cAMP levels during germination are responsible for inhibiting expression or activity of the enzyme.

The inactivation of β-glucosidase in the presence of cAMP correlates with the elevated cAMP levels in *Mucor* yeasts and the inability of these cells to utilize disaccharide carbon sources. The inability of yeasts to grow on disaccharides may be at least partially caused by a lack of active enzyme due to the elevated cAMP levels. In conjunction with this, Flores-Carreon et al. (6) have shown that maltose uptake in aerobically grown *Mucor* is dependent upon a functional respiratory chain. Therefore, both transport and hydrolysis of disaccharides may be impaired in *Mucor* yeasts.

Although cAMP in vivo results in β-glucosidase inactivation, no effect of cAMP on enzyme activity in vitro has been demonstrated in either permeabilized or disrupted cells. The mechanism of inactivation is, therefore, unknown. cAMP has been demonstrated to activate glycogen phosphorylase from mammalian sources by stimulation of cAMP-dependent protein kinase (10). Evidence exists that cAMP may be important in the control of alternate carbon source utilization in yeast. In *Saccharomyces*, a cAMP-dependent activation of trehalase has been reported (20). In addition, the α-glucosidase (22) and maltose uptake (7) systems from *Saccharomyces* are inactivated in the presence of glucose. cAMP has been shown to partially protect the α-glucosidase from destruction (22). It is possible that β-glucosidase inactivation in *Mucor* involves a phosphorylation of the enzyme. If this is the case, cAMP-dependent phosphorylation would lead to enzyme inactivation; dephosphorylation would cause reactivation.

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