Cyclic AMP, Trehalase and Germination of *Phycomyces blakesleeanus* Spores

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When heat activated *Phycomyces blakesleeanus* spores were incubated at room temperature a rapid and transient increase in cyclic AMP content was observed. A similar increase was observed during the heat treatment itself but the cyclic AMP content had returned to the value of dormant spores at the end of the 5 min treatment. The cyclic AMP content increased only after treatments at temperatures which also induced spore germination. A more gradual rise in cyclic AMP was also found during activation at room temperature with acetate and after activation of the spores with pyrosulphite. The increase in cyclic AMP content is the earliest event known to occur after activation of the spores. It clearly preceded the increase in trehalase activity observed during and/or after all the activating treatments, suggesting that, as shown for *in vitro* activation, the *in vivo* activation of trehalase is due to a cyclic AMP-dependent phosphorylation. An increase in cyclic AMP was a common feature of all activation methods investigated and appears to be the factor triggering spore germination.

INTRODUCTION

The sporangiospores of *Phycomyces blakesleeanus* do not germinate under conditions suitable for growth and development of the organism, but require activation by physical or chemical means. Heating for some minutes at about 50 °C (Robbins *et al.*, 1942) or addition of acetate (Borchert, 1962) will result in nearly 100% germination. Germination of the spores is accompanied by a decrease in trehalose content (Rudolph & Ochs, 1969), the main reserve substance of dormant spores (up to 30% of the spore dry weight). This decrease coincides with a more than tenfold increase in trehalase activity observed after activation by either heat (Van Assche *et al.*, 1972) or acetate (Delvaux, 1973). It was suggested that this trehalose breakdown was responsible for the induction of the initial steps of germination and thus for the irreversibility of the activated state (Van Laere *et al.*, 1980a). Shortly after activation the concentration of hexose monophosphates and bisphosphates increases sharply (Van Laere *et al.*, 1983) and respiration and fermentation increase dramatically. Although some treatments activate the spores without a simultaneous increase in trehalase activity (Van Assche *et al.*, 1977, 1978; Van Laere *et al.*, 1980b; Thevelein *et al.*, 1980), this activity increases rapidly during subsequent incubation in a glucose-containing medium (Thevelein *et al.*, 1983). Recently it has been shown that trehalase from dormant spores can be activated *in vitro* by a cyclic AMP- and ATP-dependent process, suggesting a phosphorylation of the enzyme (Van Laere & Hendrix, 1983). If such a mechanism also occurs *in vivo* one could expect a change in the concentration of cyclic AMP during or after the activating treatments. Such a change could also affect the degree of phosphorylation of other enzymes. Here we report the occurrence of such changes in cyclic AMP concentration and their relation with trehalase activation and spore germination.

METHODS

Organism. Strain K 1⁺ of *Phycomyces blakesleeana* Burgeff, from the collection of W. Halbsguth, was cultured on sterile moistened brown bread in the dark at 18 °C and 70% relative humidity. Spores were grown, harvested, dried and stored as described previously (Van Assche *et al.*, 1972).

Activation of the spores. Spores (10 mg ml⁻¹) were preincubated in 10 mM-KH₂PO₄ at pH 6.5 with or without 1.5% (w/v) glucose for 30 min at 25 °C. Heat activation was done by shaking this spore suspension in a water bath for 5 min at 38, 42, 46 or 50 °C. After the heat shock the suspension was immediately cooled in an ice bath and further incubated at 25 °C. For acetate activation, spores (10 mg ml⁻¹) were presoaked in water for 30 min at 25 °C, collected by centrifugation and resuspended in a medium containing 0.1 M-acetic acid and 0.01 M-H₃PO₄ brought to pH 6.5 with ammonia. Activation by pyrosulphite was done by shaking a spore suspension (10 mg ml⁻¹) in 0.1% (w/v) Na₂S₂O₅ for 15 min in a water bath at 25 °C. The spores were then collected by centrifugation, washed twice with distilled water and resuspended (10 mg ml⁻¹) in 10 mM-KH₂PO₄ with 1.5% (w/v) glucose pH 6.5.

Determination of the cyclic AMP content. At the appropriate stages of development spore suspension (2 ml, containing 20 mg spores) was rapidly filtered on Whatman glass fibre filters (GF/C, 25 mm). Within 5 s from the time the spores were removed from the culture flasks, the filters were immersed in liquid N₂. The frozen spores were homogenized in a Teflon container with 0.8 ml glass beads of 3 mm diameter and 2.2 ml glass beads of 1 mm diameter. Both container and glass beads were precooled in liquid N₂. After 3 min vibration at an amplitude of 10 mm (Braun microdismembrator), and before the contents of the container were thawed, 1 ml of a solution containing 1 M-HClO₄ and 5 mM-EDTA was added to the frozen powder of dry spores. Vibration was continued for some 3 min at the same amplitude until the contents were thawed. The homogenate was centrifuged for 2 min at 10000 g. A 0.5 ml sample of the supernatant was neutralized by adding 50 µl 5 M-K₂CO₃ and 50 µl 2 M-Tris/HCl buffer pH 7.5. The potassium perchlorate was precipitated by centrifugation for 2 min at 1000 g. A 50 µl sample of the supernatant was used for cyclic AMP determination, which was done according to Tovey *et al.* (1974), with an assay kit (Amersham). In control experiments, samples treated with phosphodiesterase (Boehringer-Mannheim) before the cyclic AMP assay yielded zero values. Addition of a known amount of cyclic AMP to some extracts yielded recoveries near 100%.

Trehalase assay and protein determination. For the determination of trehalase specific activity spore suspension (0.5 ml, containing 5 mg spores) was rapidly injected into 1 ml distilled water and centrifuged for about 10 s at 10000 g. When glucose was present in the medium, the spores were resuspended in distilled water and centrifuged for another 10 s. The spores were then quickly suspended in 0.5 ml ice-cold 10 mM-potassium phosphate buffer pH 6.5 containing 10 mM-NaF and 1 mM-phenylmethylsulphonyl fluoride, and homogenized by vortexing with 1.2 ml glass beads of 0.5 mm diameter in a test tube of 11 mm diameter for 1 min. A sample of the 2 min, 10000 g supernatant was assayed for trehalase specific activity as described previously (Thevelein *et al.*, 1983). Protein content was determined by the Lowry method. Since trehalase activity changed very quickly after heat activation we tried to minimize time delays in some experiments: a 1.0 ml sample (containing 10 mg spores) was filtered onto a Whatman glass fibre filter (GF/C, 25 mm) and submerged in liquid N₂ within 5 s. The filters were treated as for the cyclic AMP determinations except that the extraction was done in the potassium phosphate buffer described above. Since, for unknown reasons, this method yielded a much lower activity it was only used in some experiments to verify the time course of changes in trehalase activity.

Determination of percentage germination. Spore suspensions (0.2 ml, containing 2 mg spores) were centrifuged, washed with distilled water and resuspended in 1 ml minimal nutrient medium (Rudolph, 1958). Percentage germination was determined under a microscope after incubation for 6 to 8 h at 25 °C. Usually, a total of 200 to 300 spores was counted.

RESULTS

Immediately after a 5 min heat treatment (at different temperatures) the concentration of cyclic AMP was not significantly different from that in dormant spores (Fig. 1). However, the concentration of cyclic AMP increased sharply during a subsequent incubation at 25 °C, reached a maximum after 2 min and then decreased to the level found in dormant spores. The cyclic AMP content increased only when the temperature of the heat shock was high enough to induce activation of the spores. Treatment at 38 °C had no effect; after treatments at higher temperatures, the magnitude of the increase was dependent on the temperature of the heat shock. After a largely reversible activation at 42 °C, the presence of glucose in the medium prevented the spores from returning to the dormant state. The cyclic AMP concentrations measured 1, 2 and 4 min after a heat treatment at 42 °C were significantly larger ($P \leq 0.025$) in the presence of glucose than in its absence. Even after irreversible activation of the spores (e.g.

Fig. 1. Changes in cyclic AMP concentration, specific activity of trehalase and percentage germination during and after 5 min heat shocks at different temperatures in the absence (○) or presence (●) of exogenous glucose. The bars represent standard errors ($n = 3$). Do, start of heat treatment; A, end of heat treatment.

at 46 or 50 °C) the observed cyclic AMP contents were generally significantly larger when glucose was present in the medium. The highest increase (about fourfold) was obtained 2 min after a heat treatment at 50 °C in a glucose-containing medium.

Cyclic AMP concentrations were also measured during a heat shock at 50 °C. Here, too, a large increase in cyclic AMP concentration was observed 1 min after the beginning of the heat treatment. During the following 4 min, however, the cyclic AMP concentration decreased again to the level found in dormant spores. No increase in cyclic AMP content was observed during a heat treatment at 38 °C (Fig. 1).

Fig. 2

Fig. 2. Specific activity of trehalase during and after a 5 min heat shock at 50 °C in a medium without glucose as measured after two different sampling and homogenization methods. In the first method (●) the spores were centrifuged, washed and homogenized in buffer; about 40 s elapsed before homogenization. In the second method (○) the spores were collected by filtration on glass fibre filters, quickly immersed in liquid N₂ (after about 5 s) and homogenized dry and frozen before addition of the buffer (see Methods). The bars represent standard errors ($n = 3$). Do, start of heat treatment; A, end of heat treatment.

Fig. 3

Fig. 3. Changes in cyclic AMP concentration, specific activity of trehalase and percentage germination after addition of 0.1 M-ammonium acetate (indicated by the arrow) to dormant *P. blakesleeanus* spores. The bars represent standard errors ($n = 3$).

Only heat treatments which induced activation of the spores were followed by an increase in trehalase activity during subsequent incubation at 25 °C (Fig. 1). Immediately after the heat shock, the specific activity of trehalase was already higher than in dormant spores, particularly in the presence of exogenous glucose. When glucose was present in the medium, however, the spores were given an extra washing before homogenization in order to avoid high blanks in the trehalase assay. This time period may have been sufficient to allow some increase in trehalase activity. In any case, the specific activity of trehalase further increased to reach a maximum after about 4 min, thus following the maximum in cyclic AMP concentration (see also Fig. 2). Exogenous glucose enhanced the specific activity of trehalase after a heat treatment which caused a largely reversible activation of the spores. Indeed, the trehalase activities measured 1, 2

Fig. 4. Changes in cyclic AMP concentration and specific activity of trehalase during and after a 15 min treatment with pyrosulphite. The end of the pyrosulphite treatment is indicated by the arrows. The activating treatments yielded variable germination percentages ($67.1\% \pm 11.2$). The bars represent standard errors ($n = 3$).

and 4 min after a heat treatment at 42°C were significantly larger ($P \leq 0.025$) in the presence of glucose than in its absence. In contrast to its effect on cyclic AMP levels, exogenous glucose had no significant effect on the activity of trehalase after heat shocks at 46 and 50°C .

The specific activity of trehalase was also measured during a heat shock at 50°C (in the absence of glucose) (Fig. 2). Since we suspected that time delays before homogenization could allow activation of trehalase (e.g. as a result of cooling) a method was used that reduced the delay to less than 5 s (see Methods). Although this method apparently inactivated more than 50% of the trehalase, it showed that the activity of trehalase reached a maximum 2 min after the beginning of the heat treatment, paralleling the maximum in cyclic AMP concentration observed during the heat shock. The trehalase activity was again low at the end of the 5 min heat treatment.

The cyclic AMP concentration in the spores before and during incubation in 0.1 M-ammonium acetate is shown in Fig. 3. Addition of acetate induced a two- to threefold increase in cyclic AMP content as well as a sharp increase in trehalase activity during the 10 to 15 min period which was needed to activate the majority of the spores. The decrease in both cyclic AMP concentration and trehalase activity during further incubation was much slower than that after heat activation.

Treatment of *P. blakesleeana* spores with 0.1% (w/v) sodium pyrosulphite activates them without a concomitant increase in trehalase activity (Van Assche *et al.*, 1978). No significant increase in trehalase activity or in cyclic AMP concentration was observed during the incubation in pyrosulphite (Fig. 4). After the spores had been washed and incubated in a glucose-containing medium a twofold increase in cyclic AMP content and a concomitant increase in trehalase activity were observed. The fact that these effects were less pronounced than those observed for the other activation treatments could be due partly to the lower percentage germination induced by sodium pyrosulphite ($67.1\% \pm 11.2$).

DISCUSSION

The activation of trehalase has until now been the earliest event described during *Phycomyces* spore germination. However, we report here that the increase in trehalase activity after

heat activation was preceded by a clearcut increase in cyclic AMP concentration. This increase in cyclic AMP concentration was observed only after heat treatments which induced activation of the spores. Surprisingly, a very rapid increase in cyclic AMP content was also found during the heat treatment itself. Treatment with 0.1 M-acetate caused a more gradual increase in both cyclic AMP and spore germination. Since a rise in cyclic AMP content was also found after pyrosulphite activation, the increase in cyclic AMP content appears to be a common feature of early germination in *P. blakesleeanus* spores. During germination of *Blastocladiella emersonii* zoospores a maximum in cyclic AMP concentration was observed after 20 min germination (Vale *et al.*, 1976), but during germination of *Mucor* spores the increase in cyclic AMP concentration was much slower and reached a maximum only after several hours (Paznokas & Sypherd, 1975; Orłowski, 1980). During germination of *Neurospora crassa* conidia the endogenous levels of cyclic nucleotides did not change significantly (Rosenberg & Pall, 1978).

Trehalase from dormant *Phycomyces* spores can be activated *in vitro* by a cyclic AMP- and ATP-dependent process (Van Laere & Hendrix, 1983). Since the *in vivo* activation of trehalase was invariably preceded by an increase in cyclic AMP content, and since a decrease in cyclic AMP content was followed by a decrease in trehalase activity, a similar mechanism appears to operate *in vivo*. A parallel between cyclic AMP content and trehalase activity was observed during heat and acetate activation and after heat and pyrosulphite activation. Moreover, heat treatments at higher temperatures, resulting in higher cyclic AMP concentrations, were followed by higher trehalase activities (at least up to 46 °C, where saturation could occur). A similar mechanism for the *in vivo* activation of trehalase after glucose addition was inferred from results with vegetative yeast cells (Van der Plaats & Van Solingen, 1974; Van Solingen & Van der Plaats, 1975; Ortiz *et al.*, 1983) and yeast ascospores (J. Thevelein, personal communication). An increase in cyclic AMP content and trehalase activity was also found after, but not during, a heat shock of yeast ascospores (J. Thevelein, personal communication).

Changes in cyclic AMP levels and in trehalase activity induced by 0.1 M-acetate were independent of the presence of exogenous glucose. However, trehalase activation after pyrosulphite activation of the spores is largely dependent on the presence of exogenous glucose (Thevelein *et al.*, 1983). Exogenous glucose also prevented reversibly heat-activated spores from returning to the dormant state (Fig. 1; see also Van Laere *et al.*, 1980a). The irreversibility of a heat treatment at 42 °C in the presence of glucose was probably caused by the significantly higher cAMP content and/or the significantly higher trehalase activity in the presence of glucose than in its absence (Fig. 1). No such effect of glucose was found after a heat treatment at higher temperatures, where the amount of cyclic AMP could be saturating, even in the absence of glucose.

It has been proposed that plasma membrane depolarizations control cyclic AMP levels in fungi (Pall, 1977; Trevillyan & Pall, 1979; Uno & Ishikawa, 1981). Recently it has also been shown that addition of glucose and proton ionophores to yeast cells produced an immediate rise in cyclic AMP content and a concomitant inactivation of fructose-1,6-bisphosphatase (Mazon *et al.*, 1982a, b). Addition of glucose and proton ionophores to yeast cells and to yeast ascospores also caused an immediate increase in trehalase activity (J. Thevelein, personal communication). These phenomena have been explained by these authors by assuming a depolarization of the plasma membrane induced by glucose and the ionophores, although other authors believe that the effect of glucose on cyclic AMP levels in yeast is mediated by one of its catabolites (Purwin *et al.*, 1982). However, in contrast to the response in yeast, addition of glucose or proton ionophores to dormant *P. blakesleeanus* spores was not followed by an increase in cyclic AMP concentration nor in trehalase activity (not shown).

Despite its transience, the increase in cyclic AMP concentration could induce, via phosphorylation, dramatic increases in the activity not only of trehalase but also of other key regulatory enzymes. Since this increase is a common feature of different activation procedures, cyclic AMP might prove to be the signal controlling the relief of dormancy.

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