SHORT COMMUNICATION

Cyclic AMP-dependent *in vitro* Activation of Trehalase from Dormant *Phycomyces blakesleeanus* Spores

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The activity of trehalase in a crude extract of dormant *Phycomyces blakesleeanus* spores increased 10- to 20-fold during incubation in the presence of cyclic AMP and ATP. A phosphorylation of the enzyme was probably involved, as a more pronounced activation was obtained when cyclic AMP was replaced by the catalytic subunit of beef heart protein kinase. The γ -thio derivative of ATP could replace ATP in the activation but was quantitatively less effective. The effective concentrations of cyclic AMP and ATP were comparable to their likely intracellular concentrations; thus phosphorylation could be the *in vivo* mechanism of trehalase activation during the activation of dormant spores by heat or acetate.

INTRODUCTION

Trehalose is a common reserve carbohydrate in fungal spores and trehalase breakdown during germination has been documented in several species. In some cases the breakdown of trehalose has been related to increases in trehalase activity, such as in *Dictyostelium discoideum* (Cotter & Raper, 1970), *Aspergillus oryzae* (Horikoshi & Ikeda, 1966) and *Phycomyces blakesleeanus* (Van Assche *et al.*, 1972). The most striking effect has been described for the constitutively dormant sporangiospores of *P. blakesleeanus*. Trehalose constitutes up to 30% of the dry weight of these spores and is broken down during early germination (Rudolph & Ochsén, 1969). This breakdown has been correlated with a more than tenfold increase in trehalase activity during activation of the spores by both heat (Van Assche *et al.*, 1972) and acetate (Delvaux, 1973) treatment. Increase in trehalase activity was shown to be independent of the synthesis of new protein (Van Assche *et al.*, 1972). A thermal transition in a surrounding polysaccharide matrix has been suggested as the factor mediating the increased activity (Thevelein *et al.*, 1981) but the actual mechanism remains unknown. Rapid changes in trehalase activity have also been described in *Saccharomyces cerevisiae* (Van der Plaats & Van Solingen, 1974) and have been correlated with a cyclic AMP-dependent phosphorylation of the enzyme (Van Solingen & Van der Plaats, 1975; Ortiz *et al.*, 1983). Here we report similar effects obtained with trehalase from dormant *P. blakesleeanus* sporangiospores.

METHODS

Organism. All experiments were done with the sporangiospores of the K 1⁺ strain of *Phycomyces blakesleeanus* Burgeff, prepared as before (Van Assche *et al.*, 1972).

Preparation of crude extracts. Dormant spores (100 mg) were homogenized in 1.5 ml ice-cold 10 mM-potassium phosphate buffer pH 6.5 (Thevelein *et al.*, 1983). The crude extract was centrifuged for 15 min at 100000 g (0-5 °C). For the experiments described in Fig. 1 and Table 2 the supernatant was desalted on a column of Sephadex G-25 equilibrated with extraction buffer.

***In vitro* activation of trehalase.** Except when stated otherwise, the crude extract (containing between 3 and 5 mg protein ml⁻¹) was incubated at 25 °C in the presence of 1.8 mM-MgSO₄, 0.8 mM-ATP, 10 μ M-cyclic AMP, 1 mM-

Abbreviations: PMSF, phenylmethyl sulphonylfluoride; ATP- γ -S, γ -thio derivative of adenosine 5'-triphosphate.

theophylline, 0.2 mM-PMSF and 10 mM-NaF. In some experiments ATP was replaced by ATP- γ -S (Serva, Heidelberg). Some incubations were done with cyclic AMP replaced by the catalytic subunit of beef heart protein kinase (Sigma).

Trehalase assay. Trehalase activity was measured by addition of 20 μ l amounts of the cell-free extract to 230 μ l reaction mixture containing 40 mM-trehalose and 40 mM-phosphate buffer pH 7.5 (final concentrations). Samples were boiled after incubation at 30 °C for 0 min (blank value) and 15 min. The glucose liberated was measured by the glucose oxidase-peroxidase method (Lloyd & Whelan, 1969). Protein content was determined by the Lowry method. Specific activity is expressed as nmol glucose liberated min^{-1} (mg protein) $^{-1}$. Since specific activities and activation rates were somewhat variable between different extracts, data presented in each table or figure were obtained with the same extract. All experiments were repeated with generally consistent results.

RESULTS

The specific activity of trehalase in a crude extract of dormant spores of *P. blakesleeenanus* was very low, about 2 nmol min^{-1} (mg protein) $^{-1}$. The tenfold increase in activity caused by a 15 min

Table 1. *Specific activity of trehalase in a crude extract of dormant P. blakesleeenanus spores incubated for 15 min at 25 °C in 10 mM-phosphate buffer pH 6.5 with various additions*

Incubation mixture*	Specific activity [nmol glucose min^{-1} (mg protein) $^{-1}$]	Activation
Control (before incubation)	2.0	[1.0]
+ cAMP	3.0	1.5
+ MgATP	2.0	1.0
+ MgATP + cAMP	25.1	12.5
+ MgATP + cAMP + PMSF	28.1	14.0
+ MgATP + cAMP + fluoride	35.6	17.8
+ MgATP + cAMP + theophylline	37.9	18.9
Complete mixture	46.1	23.0
Complete mixture - PMSF	39.1	19.5
Complete mixture - fluoride	35.2	17.6
Complete mixture - theophylline	33.4	16.7
Complete mixture - ATP	3.1	1.5
Complete mixture - cAMP	2.4	1.2

* See Methods for concentrations.

Fig. 1. Increase of trehalase activity during incubation of a desalted extract of dormant *P. blakesleeenanus* spores at 25 °C in a complete activation mixture with 0.8 mM-ATP (○, ●) or ATP- γ -S (□, ■). Closed symbols, with 10 μ M-cAMP; open symbols, without cAMP but with catalytic subunit of beef heart protein kinase (10 μ g ml^{-1}).

Table 2. *Effect of different ATP and cAMP concentrations on the activation of trehalase in a desalted extract of dormant P. blakesleeanus spores during a 15 min incubation at 25 °C*

Addition(s)	Specific activity [nmol glucose min ⁻¹ (mg protein) ⁻¹]	Activation
None	1.2	[1.0]
0.8 mM-ATP	1.3	1.1
0.8 mM-ATP + 1 µM-cAMP	13.8	11.6
0.8 mM-ATP + 2 µM-cAMP	16.3	13.7
0.8 mM-ATP + 10 µM-cAMP	19.9	16.8
10 µM-cAMP	1.2	1.0
10 µM-cAMP + 0.32 mM-ATP	7.9	6.7
10 µM-cAMP + 0.8 mM-ATP	16.0	13.5
10 µM-cAMP + 1.6 mM-ATP	18.9	15.9

preincubation of a concentrated extract in the presence of cyclic AMP, ATP and Mg²⁺ at 25 °C was completely dependent on both cyclic AMP and ATP. The extent of activation could be increased a little by addition of the protease inhibitor PMSF and more by fluoride and theophylline (inhibitors respectively of phosphatases and phosphodiesterases) (Table 1).

The specific activity of trehalase increased up to 40 min during preincubation with a complete activation mixture with endogenous activating factors (Fig. 1). Replacement of ATP by ATP-γ-S diminished activation by about 40%, while replacing cyclic AMP by the catalytic subunit of beef heart protein kinase approximately doubled activation (Fig. 1).

Activation depended on the concentrations of ATP and of cyclic AMP added (Table 2). Although in the crude extract used the actual concentrations may have decreased considerably during incubation, the concentrations where activation was obtained were clearly in the physiological range.

DISCUSSION

The activation of trehalase in extracts of *P. blakesleeanus* spores by cyclic AMP and ATP suggests that the enzymic activity can be regulated by phosphorylation and dephosphorylation, the phosphorylated enzyme being 10 to 20 times more active. The actual degree of activation largely depended on the initial specific activity of the extract, which varied between 1 and 3 nmol min⁻¹ (mg protein)⁻¹ in different extracts. The involvement of phosphorylation was also suggested by the even greater activation observed when the catalytic subunit of beef heart protein kinase replaced cyclic AMP. ATP could be replaced by its γ-thio analogue with up to 40% reduction in the observed activation. Whether this was due to a slower rate of activation or to a smaller increase in activity upon thiophosphorylation could not yet be distinguished. Besides the longer half-life of radioactively-labelled thioderivatives, thiophosphorylation could have experimental advantages by increasing the resistance of the thiophosphorylated trehalase to phosphatase (Cassidy *et al.*, 1979) and allowing isolation of the thiophosphorylated protein by mercury affinity chromatography (Sun *et al.*, 1980).

The comparable degree of trehalase activation observed here *in vitro* and in response to activation of *P. blakesleeanus* spores by heat (Van Assche *et al.*, 1972) or acetate (Delvaux, 1973) suggests that the two processes could have a similar mechanism. Although we could find no increase in total cyclic AMP after heat activation of the spores (results not shown), *in vivo* phosphorylation could perhaps be stimulated by an increase in free cyclic AMP or by a direct activation of protein kinase (e.g. heat-induced dissociation of a regulatory subunit).

Trehalase activation can be uncoupled from spore activation by a variety of treatments (Van Assche *et al.*, 1978; Van Laere *et al.*, 1980; Thevelein *et al.*, 1981) but an increase in trehalase activity is invariably associated with early germination (Thevelein *et al.*, 1983). Therefore a change in the degree of phosphorylation of trehalase and possibly other enzymes could be one of the signals directing germination of the spores.

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