Cyclic AMP signalling pathway and trehalase activation in the fission yeast *Schizosaccharomyces pombe*

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**INTRODUCTION**

The metabolic response of derepressed cells of *Saccharomyces cerevisiae* to glucose involves a cyclic AMP (cAMP) signalling pathway which has been the subject of considerable attention (for reviews, see Thevelein, 1991, 1992). Genetic and biochemical evidence indicates that *RAS1* and *RAS2* gene products, which are the yeast homologues of the mammalian RAS proteins, mediate a transient activation of adenylate cyclase in response to stimulation by glucose (Gibbs & Marshall, 1989). The increased level of cAMP promotes binding of the nucleotide to the regulatory subunit of cAMP-dependent protein kinase causing its dissociation from the catalytic subunits which are thus activated. Further transmission of the cAMP signal involves covalent modification by phosphorylation of various key enzymes in carbon metabolism (Wills, 1990).

Among fungi, the existence of glucose-induced cAMP signalling has been reported in several zygomycetes (Dewerchin & van Laere, 1984; van Mulders & van Laere, 1984) and suspected in budding yeasts related to *S. cerevisiae* (Arguelles & Gacto, 1985). In particular, the occurrence of this signal in the fission yeast *Schizosaccharomyces pombe* has been questioned because there is clear evidence that the single RAS protein present in this yeast does not act as a regulator of adenylate cyclase (Fukui et al., 1986; Thevelein, 1991). However, recent results by Byrne & Hoffman (1993) provide evidence for a glucose-induced cAMP signalling pathway in this yeast that appears to be required for glucose repression of the *fhp1* gene.

In this context, previous studies from this laboratory have shown that the addition of fermentable sugars, protonophores or uncouplers to derepressed cells of *Sr. pombe* results in activation of the neutral trehalase (EC 3.2.1.28) and that this stimulation is an energy-requiring post-translational event which can also be triggered both in repressed and derepressed growing cells by adding cAMP to the incubation buffer (Carrillo et al., 1992). In the present report, we show that the increase in trehalase activity which occurs after addition of the above inducers is indeed preceded by a rapid increase in intracellular cAMP. The role of cAMP in the modulation of trehalase activity in cells under heat shock treatment has also been examined.

**Keywords**: *Schizosaccharomyces pombe*, trehalase activation, cyclic AMP, signalling pathways
Yeast strains and growth conditions. The wild-type strain *Schizosaccharomyces pombe* 927h" was cultured at 28 °C with shaking in a liquid medium containing 2% (w/v) glucose and 0.3% yeast extract. The cells were collected by centrifugation from derepressed stationary-phase cultures after glucose exhaustion (OD<sub>600</sub> = 4.5). Alternatively, the organism was grown on 2% (w/v) glycerol and 0.6% yeast extract and harvested from derepressed cultures in the exponential phase of growth (OD<sub>600</sub> = 1.0). The cells were washed with cold distilled water and resuspended in 10 mM MES/KOH buffer, pH 6.0, at a concentration of 50 mg wet weight ml<sup>-1</sup> (approximately 8 × 10<sup>8</sup> cells ml<sup>-1</sup>). In comparative experiments *Saccharomyces cerevisiae* X2180-1A was cultured in the glucose medium described above, the cultures recovered at stationary phase (OD<sub>600</sub> = 5.0) and the cells obtained and processed as indicated.

Activation of trehalase and preparation of cell extracts. Suspensions of derepressed cells were incubated in a shaking water bath and allowed to equilibrate at 30 °C for 5 min. Before and after addition of the compounds assayed (zero time), samples (1.5 ml) were removed at timed intervals and the cells washed as above. To each final pellet, 0.7 ml 10 mM MES/KOH buffer, pH 6.0, was added and the cells were broken using a vortex mixer with glass beads (0.5 mm diameter) and intermittent cooling in an ice bath. The cell extracts were then centrifuged in the cold for 5 min at 12000 g in an Eppendorf microfuge and the resulting supernatant fluids were used for enzyme assays and protein determination. In the case of experiments with repressed cells, to ensure repressive conditions, cultures growing exponentially on glucose (OD<sub>600</sub> = 0.2) were directly supplemented with the compound to be assayed instead of performing the addition to the cell suspension maintained in buffer. At the time stated in the text, samples (70 ml) were obtained and the cells were washed and mechanically disrupted as indicated for derepressed cells.

For activation of trehalase upon heat shock, the procedure of De Virgilio et al. (1990) was followed. Exponential-phase cultures (OD<sub>600</sub> = 0.2) growing on glucose medium were shifted at 40 °C, whereas parallel control cultures were maintained at 28 °C.

Trehalase assay and protein determination. Reaction mixtures for trehalase assay contained 0.6-0.9 mg protein, 100 mM trehalose and 10 mM MES/KOH, pH 6.0, in a final volume of 0.5 ml. After incubation for 10-30 min at 30 °C the reaction tubes were immersed in a boiling water bath for 2 min, cooled and centrifuged at 3000 g for 10 min. Glucose was determined in the supernatant fluids as described elsewhere (Arguelles & Gacto, 1985). One unit of trehalase released 1 nmol of glucose min<sup>-1</sup> under these conditions. Protein content was determined according to the Lowry method.

cAMP determination. cAMP levels and trehalase activity were always determined simultaneously on the same cultures. At the times indicated before and after addition of the various compounds assayed, 250 μl of the cell suspension (about 2 × 10<sup>8</sup> cells) was withdrawn and transferred into precooled tubes containing the same volume of 1 M trichloroacetic acid (TCA). The samples were immediately frozen in liquid nitrogen, freeze-thawed four times and then centrifuged in the cold at 12000 g for 3 min. The supernatants were extracted three times with 4 vols water-saturated ethyl ether to eliminate TCA from the extracts and thereafter they were freeze-dried, dissolved in 50 μl 50 mM Tris/HCl, pH 7.5, supplemented with 4 mM EDTA and assayed for cAMP content using the Amersham (³H) cAMP radioassays kit according to the supplier's instructions. cAMP concentrations were determined within the recommended range of maximum precision and expressed as pmol (g wet weight)<sup>-1</sup>. When this same procedure of cAMP determination was applied to *S. cerevisiae* X2180-1A for comparative purposes, the values obtained after addition of glucose were comparable to those described by Arkinstall et al. (1991) for several strains of this yeast.

Reproducibility of results. All experiments were repeated at least three times with similar results. Representative results are shown.

RESULTS

We have shown previously that addition of glucose to derepressed cells of *S. pombe* provokes a reversible activation of neutral trehalase (Carrillo et al., 1992). In contrast, the addition of the inhibitor of protein synthesis cycloheximide, the protonophore 2,4-dinitrophenol or the uncoupler sodium azide to derepressed cells did not cause, by itself, changes in trehalase activity (Fig. 1a). However, each of these compounds produced a marked stimulation of enzyme activity in the presence of glucose.
cAMP and trehalase in *Sc. pombe*

Fig. 2. Effect of the addition of 100 mM glucose on trehalase activity (□) and cAMP levels (○) in derepressed cells grown on glycerol. Glucose was added at zero time and trehalase and cAMP determined as described in Methods. The inset shows the consumption of the glucose (Glc) added to the cell suspension. Trehalase and cAMP in control cells are represented by the discontinuous line.

Fig. 3. Effect of the addition of 100 mM glucose to derepressed cells from stationary phase cultures grown on glucose. Symbols for trehalase, cAMP and glucose consumption are as indicated in the legend to Fig. 2.

and was able to reactivate trehalase after a first activation-deactivation process induced by the sugar (Fig. 1a).

When similar experiments were performed with repressed cells, the activity of the trehalase enzyme in cultures supplemented with glucose was almost identical to that of control cultures, indicating that glucose was unable to increase trehalase activity in repressed cells (Fig. 1b). Confirming earlier results by De Virgilio et al. (1991), we found about the same basal values for neutral trehalase activity during exponential growth on glucose and in stationary phase (Fig. 1a, b) so that the lack of activation of trehalase in repressed cells cannot be interpreted as due to a previous full activation maintained by glucose. The enzyme activity also was unaffected in repressed cells by sodium azide, whereas after addition of cycloheximide or 2,4-dinitrophenol an increase was noticed (Fig. 1b).

In *S. cerevisiae* trehalase is regulated by cAMP-dependent protein phosphorylation (Uno et al., 1983). Since the existence of a cAMP signalling pathway in *Sc. pombe* has been recently proposed (Hoffman & Winston, 1991; Byrne & Hoffman, 1993), it seemed worthwhile to examine possible changes in the intracellular cAMP content under the different conditions in which trehalase was activated in these cells. The results indicated that the addition of glucose to derepressed cells of the fission yeast causes a rapid and pronounced increase in the intracellular level of cAMP prior to trehalase stimulation. This effect was clearly shown both in cells from cultures growing on glycerol (Fig. 2) and in cells from stationary-phase cultures after growth on glucose (Fig. 3). In contrast to what has been described for the budding yeast *S. cerevisiae* (Thevelein & Beullens, 1985) neither the glucose-induced cAMP signal nor the subsequent activation of the trehalase enzyme were abolished by the presence of acridine orange in the fission yeast (Fig. 4). On the other hand, the addition of sodium azide to derepressed cells...
Fig. 5. Changes in trehalase activity (■, ●) and corresponding levels of cAMP (○, ●) upon addition at zero time of 200 mM glucose to a suspension of derepressed cells from stationary-phase cultures (open symbols) and subsequent re-addition 60 min later (filled symbols) of 2 mM sodium azide (a) or 0.35 mM cycloheximide (b). At the time of the re-additions the glucose content was about 30 mM in both cell suspensions. Trehalase and cAMP levels in control cells are represented by the discontinuous line.

Changes in trehalase activity and corresponding cAMP levels in control cells during a heat shock. Exponential-phase cultures growing on glucose were shifted at zero time to 40 °C (filled symbols) or maintained at 28 °C as a control (open symbols). Trehalase and cAMP levels in control cells are represented by the discontinuous line.

under conditions which trigger trehalase activation (i.e. with glucose present in the incubation buffer) was also accompanied by a rise in the cAMP level preceding the activation of the enzyme (Fig. 5a). A comparatively smaller, but reproducible increase in cAMP also was detected when cycloheximide was added as an alternative inducer (Fig. 5b). Remarkably, both the peak in cAMP and the activation of trehalase were absent when these compounds were added in the absence of glucose. Similar results were obtained when 2,4-dinitrophenol was used to induce trehalase activation (not shown).

When repressed cells of *S. pombe* growing on glucose-containing medium were further supplemented with glucose or sodium azide, no changes in the basal level of cAMP were observed, indicating that not only trehalase activation but also the cAMP pulse promoted by these inducers is sensitive to glucose repression. This result supports the concept of a common link between the two glucose-induced effects. However, within seconds of adding cycloheximide or 2,4-dinitrophenol to repressed cultures, the cells showed increases in cAMP content which were comparable to those illustrated in Fig. 5. Thus, a close correlation between increase in cAMP content and trehalase activation was also established in repressed cells.

The activation of neutral trehalase in heat-shocked cells of *S. pombe* maintained under repression conditions has been described previously (De Virgilio et al., 1990, 1991). In view of the above precedents, we next examined a possible correlation between cAMP increase and trehalase activation under these conditions. As indicated in Fig. 6, repressed cells growing exponentially on glucose markedly increased trehalase activity for at least 90 min upon exposure of the culture to 40 °C. However, no changes in cAMP content were observed suggesting the existence of an alternative, cAMP-independent pathway responsible for heat shock activation of trehalase (Fig. 6).

**DISCUSSION**

In *S. pombe* cAMP has been implicated in glucose-repression of transcription of the *fbpl* gene which encodes fructose-1,6-bisphosphatase (Hoffman & Winston, 1991). This previous evidence, and more recent results published while this study was in progress (Byrne & Hoffman, 1993), suggest the existence in this yeast of a cAMP signalling pathway independent of ras and working by activation of cAMP-dependent protein kinase. The measurement of cAMP levels performed by us in derepressed cells immediately after glucose addition supports the conclusion of Byrne & Hoffman (1993) although, the alteration in cAMP content in response to glucose was a much more rapid event in our hands. In addition, our present work shows that, as in the budding yeast *S. cerevisiae*, the rapid and reversible increase in the cAMP content can be achieved not only by glucose, but also by the inhibitor of protein synthesis cycloheximide, the protonophore 2,4-dinitrophenol or the uncoupler sodium azide which share the ability to trigger trehalase activation. The link between the changes in cAMP induced by these compounds and the parallel, although temporally retarded, activation of trehalase does not stand merely on circumstantial evidence. Taken together with results
reported earlier, demonstrating that exogenously added cAMP can activate trehalase (Carrillo et al., 1992), our data clearly indicate that glucose, and other inducers, trigger a cAMP signalling pathway which in turn can produce trehalase activation as a final effect. To our knowledge this is the first direct indication of a cAMP signal in the fission yeast able to play a post-translational role by modulating an enzyme activity.

We found that cells grown under repressed and derepressed conditions possess similar steady-state levels of cAMP (see Figs 5 and 6 for basal contents). This finding agrees with results presented by Hoffman & Winston (1991) and differs strongly from earlier data from Schlanderer & Dellweg (1974) which indicated a lower cAMP content in repressed cells. In any case, our data on the analysis of the response to glucose in both types of cells reveals differential behaviour. Only derepressed cells show altered cAMP levels and trehalase activation in response to glucose, whereas repressed cells lack the glucose-induced, transient cAMP signal and the subsequent trehalase activation.

We have previously demonstrated that the effect of exogenous cAMP on trehalase activation is not sensitive to glucose repression (Carrillo et al., 1992). In contrast to the cAMP-induced response, the glucose-induced trehalase activation appears to be under glucose catabolite repression. These results point to the existence of some glucose-repressible protein along the signalling pathway implicated in the mechanism of trehalase activation induced by glucose. Thus, the cAMP signalling pathway in *S. pombe* appears similar in design to that of *S. cerevisiae*, where the participation of a glucose-repressible protein in the glucose-induced signal has been suspected (Thevelein, 1991, 1992). Sodium azide, in contrast to cycloheximide or 2,4-dinitrophenol, does not trigger the cAMP signal in repressed cells although, as with glucose, it does have a triggering effect in derepressed cells (Fig. 1). Most likely, this compound activates the signalling pathway in an analogous way to glucose by interacting with some initial factor upstream of the putative glucose-repressible protein. On the contrary, both cycloheximide and 2,4-dinitrophenol, which apparently do not require the function of such an element, would activate a common part of the sensing system downstream of the glucose-repressible protein. This would explain their ability to activate trehalase even in repressed cells. Hence, as in budding yeasts (Thevelein, 1991, 1992), the results support the outline of a branched cAMP signalling pathway leading to trehalase activation throughout several different entries. The fact that, in all cases, the cAMP rise and the corresponding enzyme activation provoked by these compounds required the simultaneous presence of glucose probably indicates the need for available ATP as substrate for adenylate cyclase. In this context, it should be mentioned that *S. pombe* lacks the glyoxylate cycle and therefore the cells show a greatly reduced ATP content in the absence of glucose or an alternative energy source (McDonald & Tsai, 1989).

*Sc. pombe* differs profoundly from *S. cerevisiae* in many biochemical aspects (Russell & Nurse, 1986). In the fission yeast, for instance, RAS proteins do not act as regulators of adenylate cyclase (Fukui et al., 1986). Moreover, in spite of structural analogies (Yamawaki-Kataoka et al., 1989), functional differences between the adenylate cyclases in both yeasts have been described (Engelberg et al., 1990). Thus, it would not be surprising if, besides a common final effect and the participation of a glucose-repressive protein and cAMP in the two cases, the modulation of trehalase by glucose signal transduction would follow a somewhat different pathway in the two yeasts. Our results on the effect of acridine orange, which inhibits both the cAMP signal and the glucose-induced trehalase activation in the budding yeast (Thevelein & Beuclens, 1985), but not in *S. pombe*, are likely to reflect some peculiarity in the composition of the pathway present in the fission yeast additional to this RAS-independent character. A particularly intriguing question to be resolved is the nature of the event that gives rise to trehalase activation by this pathway. The simplest hypothesis to explain this would be a direct phosphorylation of the enzyme protein by cAMP-dependent protein kinase, as in the case of *S. cerevisiae* (Uno et al., 1983; Ortiz et al., 1983). Results supporting the idea that the glucose-induced trehalase stimulation in *S. pombe* is in fact due to a post-translational covalent modification of the enzyme have been presented previously (Carrillo et al., 1992). However, as reported by others (De Virgilio et al., 1991), we also have been unable to induce in vitro activation of trehalase under conditions in which trehalases from *S. cerevisiae* and Candida utilis become phosphorylated and thereby activated (Ortiz et al., 1983; Arguelles & Gacto, 1985). In addition, treatment of trehalase from the fission yeast with alkaline phosphatase does not result in changes in enzyme activity (unpublished results). Thus, although the activation appears to be mediated by cAMP-dependent protein kinase, the exact requirements for trehalase modification by this pathway remain unknown.

From our results on the activation of trehalase by heat shock (Fig. 6), it can be deduced that such a response does not follow the same pathway that is functional in the glucose response. No peak in the acid-extractable cAMP pool is produced by thermal treatment prior to the stimulation of trehalase, suggesting that the mechanism which modifies trehalase activity takes place by means of a signal in which cAMP may not be involved as a second messenger. Hence, in *S. pombe* the cAMP signalling pathway appears to exist in parallel to other pathways that can transmit the same final effect of trehalase activation. A similar situation has been shown recently for nutrient-induced activation of neutral trehalase in starved cells of *S. cerevisiae* (Hirimburegama et al., 1992). The demonstration in the fission yeast of several pathways controlling trehalase, and probably other enzymes, raises new questions related to the physiological relevance of these responses, the identification of key components and the interaction among the different elements involved in such pathways.

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