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Effects of heat shock on the level of trehalose and glycogen, and on the induction of thermotolerance in *Neurospora crassa*

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Neurospora crassa conidiospore germlings exposed to a heat shock (30–45°C) rapidly accumulated trehalose and degraded glycogen, even in the presence of cycloheximide. This phenomenon was also rapidly reversible upon return of the cells at 30°C. Trehalose accumulation at 45°C demanded an exogenous source of carbon and either glucose or glycerol fulfilled such requirement. Experiments with the cyclic AMP-deficient *cr-1* mutant suggested that the effects of temperature shifts on trehalose level were independent of cAMP metabolism. Cells exposed at 45°C under conditions permissive for trehalose accumulation (i.e. in the presence of an assimilable carbon source) also acquired thermotolerance.

Heat shock; Thermotolerance; Trehalose; Glycogen; Cyclic AMP; *Neurospora crassa*

1. INTRODUCTION

The disaccharide trehalose (1-α-D-glucopyranosyl-1,1-D-α-glucopyranoside) is ubiquously distributed in nature [1]. In fungi trehalose accumulates preferentially in spores, and it is generally admitted that spore-stored trehalose is used as a carbon and energy source for the early steps of germination [2,3]. In the yeast *Saccharomyces cerevisiae* [4–6] and *Schizosaccharomyces pombe* [7] accumulation of trehalose can be induced by heat shock and other environmental stresses including heavy metals, oxidants and organic solvents [5–9]. This phenomenon is reversible and trehalose is rapidly degraded upon return of the cells to physiological conditions. From these observations, it is now suggested that the main role of trehalose in yeast might not be a role as carbon and energy reserve but rather as a protectant against environmental injuries [7].

In the filamentous fungus *N. crassa* ascospores [10] and conidiospores [11] contain 14% and 10% of the dry weight in trehalose, respectively. The concentration of trehalose decreases to a minimal value upon germination, remains low during the vegetative growth phase and rises again at the end of growth, when it accumulates in conidiospores [11]. Other conditions for induction of trehalose accumulation in this organism have not been described up to now. In the present study we investigated the effects of heat shock on the level of the two main reserve carbohydrates of *N. crassa*: trehalose and glycogen and its consequences on the survival of cells exposed to a lethal treatment.

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2. MATERIALS AND METHODS

2.1. Neurospora *strains and culture conditions*

N. crassa wild-type (FGSC 424) and *cr-1* (crisp) mutant (FGSC 488, allele no. B-123) were a gift from the Fungal Genetics Stock Center (Kansas City, KS, USA). These strains were maintained by weekly transfers on slants of Vogel's [12] medium solidified with 1.5% agar and supplemented with 2% sucrose.

2.2. *Heat shock conditions and test of thermotolerance*

Conidiospores harvested from 8–10-day-old slants were suspended in liquid Vogel's medium (concentration 1–3 × 10^7 conidia/ml) supplemented with the carbon source indicated for each experiment. The cultures were incubated at 30°C with agitation (120 rpm) for 5 h. After that, each culture was divided in two. One-half remained at 30°C while the other was incubated at 45°C (heat-shocked culture). Aliquots of each culture were removed at pre-determined intervals and processed for determination of trehalose, glycogen and protein. The test of thermotolerance was carried out at 55°C essentially as described by Cruz et al. [13].

2.3. *Determination of trehalose, glycogen and protein*

For the determination of trehalose and glycogen 50–70 mg (wet weight) of cells were resuspended in 1.5 ml of 0.25 M Na_2CO_3 and incubated for 20 min in a boiling water bath. After that, an aliquot of the suspension was withdrawn, centrifuged and the supernatant was used for determination of trehalose after adjusting to pH 5.5 with 1 N acetic acid. The rest of the suspension remained at 100°C up to two hours, and then was processed for the enzymatic determination of glycogen according to Becker [14]. Trehalose was determined enzymatically with a trehalase preparation obtained from *Humicola grisea* [15]. The assay was carried out in a total volume of 0.4 ml in 75 mM sodium acetate buffer, pH 5.5, containing 7.5 mM $CaCl_2$ and 200 μl of the trehalose extract. The reaction was started with 2 U of the *Humicola* trehalase preparation. Incubation was carried out at 40°C for 45 min and was stopped by incubating the

samples at 100°C for 10 min. The glucose liberated was estimated by the glucose oxidase procedure.

Protein was assayed by the method of Lowry et al. with bovine serum albumin as standard.

2.4. *Chemicals*

Adenosine 3,5-cyclic monophosphate (cyclic AMP) was purchased from Sigma Chem. Co. (USA). Trehalose was a product from Merck (Germany). The glucose formed by hydrolysis of trehalose and glycogen was estimated by the glucose oxidase procedure using a commercial assay kit (Labtest, Brazil). All other chemicals were of analytical grade.

3. RESULTS AND DISCUSSION

N. crassa germlings incubated at 45°C accumulated trehalose and degraded glycogen (Fig. 1A and B). The concentration of trehalose increased during the first 30–40 min of exposure at this temperature and stabilized thereafter. After returning the heat-shocked cultures to physiological conditions (30°C), trehalose and glycogen levels changed again, approaching the initial values in about 90 min. The effects of temperature shifts on the level of both carbohydrates were not abolished by inhibition of protein synthesis. Nevertheless, in the presence of cycloheximide, the maximum levels of trehalose and glycogen were somewhat lower than in its absence.

It was of interest to know whether trehalose accumulation at 45°C would be affected in the adenylate cyclase-deficient *cr-1* (crisp) mutant [16]. This strain, as a consequence of its reduced intracellular levels of cAMP exhibits a number of morphologic, enzymatic and nutritional defects which become cured if the culture medium is supplemented with cAMP [17–19]. As shown in Fig. 2, the temperature-induced changes in the level of trehalose were identical for the mutant and the wild-type. Furthermore, addition of exogenous cAMP (5.0 mM) to cultures of either strain (not shown), was of no consequence. On the other hand, the level of glycogen of the mutant was only slightly affected by the temperature shifts. Furthermore, the results shown in Fig. 2 demonstrated that the accumulation of glycogen occurring after the heat-shocked cells were reincubated at 30°C, was dependent on the existence of an exogenous carbon source, because it was not observed for cells resuspended in carbon-free medium. Under this latter condition, even the degradation of a large pool of trehalose (Fig. 2A) did not prevent a pronounced fall in glycogen level. A significant difference observed between the wild-type and the mutant was in the basal level of trehalose, which was much higher for the mutant. This observation may explain the superiority of *cr-1* in terms of tolerance to high temperatures, as reported earlier [13] and also would suggest that levels of trehalose may be indirectly affected by cAMP.

The following experiments were aimed at investigating the source of glucose units which supported

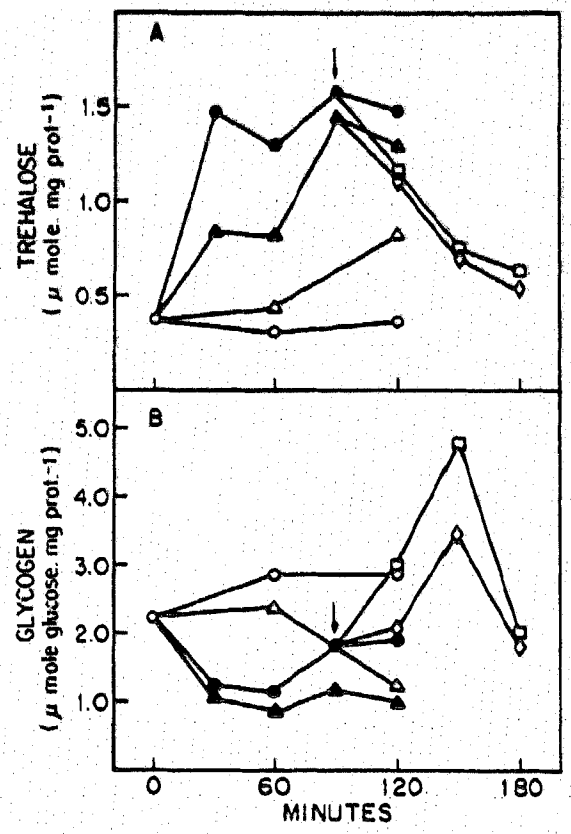

Fig. 1. Changes in trehalose and glycogen levels elicited by temperature shifts in the presence or absence of cycloheximide. *N. crassa* conidiospores were germinated for 5 h at 30°C, in Vogel's minimal medium supplemented with 2% glucose. After that time the cultures were divided and reincubated at 30°C (○, △) or 45°C (●, ▲), in the presence (△, ▲) or absence (○, ●) or 100 μg/ml cycloheximide. At the times indicated by arrows, aliquots of the cultures incubated at 45°C were returned to 30°C, with addition (◇) or not (□) of cycloheximide. At the times represented in the abscissa aliquots of the cultures were sampled and processed for determination of trehalose (A), glycogen (B) and protein.

trehalose synthesis at 45°C. Conidiospores were initially germinated in media supplemented with either 2% glucose or 1% glycerol. After the period of germination, aliquots from each primary culture were harvested and resuspended in fresh media supplemented with either 2% glucose or 1% glycerol and were reincubated at either 30°C or 45°C, such that samples from all combinations of glucose or glycerol supplementation were subjected to both temperature treatments (Fig. 3). Glucose-germinated cells accumulated trehalose at 45°C when resuspended in glucose-supplemented medium, but failed to do it in the presence of glycerol (Fig. 3A) because of repression on the enzymes required for glycerol assimilation and for gluconeogenesis [20]. On the other hand, cells germinated in the presence of glycerol and therefore adapted to glycerol metabolism, accumulated trehalose at 45°C either in the presence of glucose or glycerol (Fig. 3C), indicating

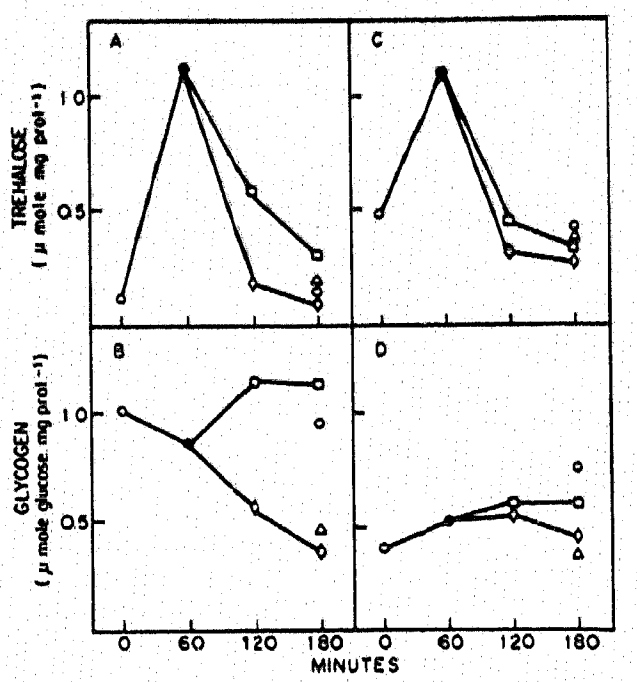

Fig. 2. Changes in the levels of trehalose and glycogen elicited by temperature shifts in the wild-type strain and the adenylate cyclase-deficient *cr-1* (crisp) mutant. Conidiospores of the wild-type (A,B) and the mutant (C,D) were germinated and submitted to heat shock as described in the legend to Fig. 1. After the heat treatment the cultures were reincubated at 30°C in Vogel's minimal medium supplemented (□) or not (◇) with 2% glucose. Other symbols: (○) cultures at 30°C; (●) cultures at 45°C; (△) cultures maintained at 30°C in glucose-free medium. At the times represented in the abscissa aliquots of the cultures were sampled and processed for determination of trehalose (A,C) and glycogen (B,D).

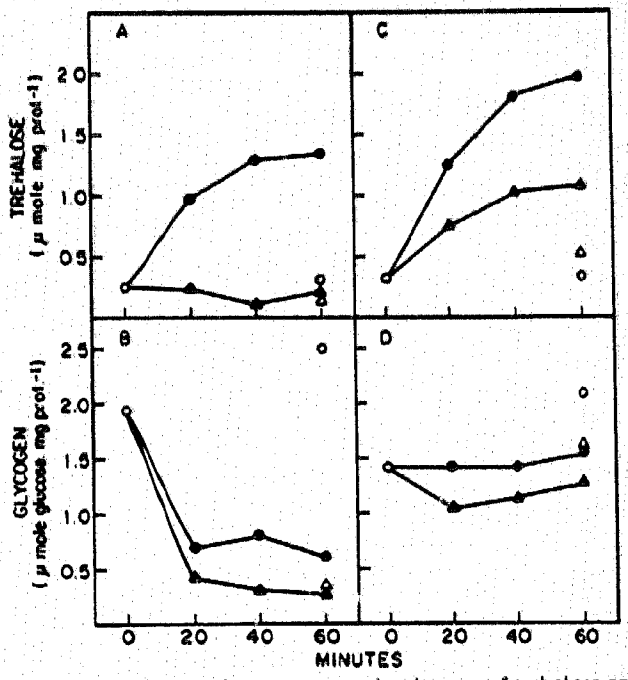

Fig. 3. Effect of the carbon source on the changes of trehalose and glycogen elicited by heat shock. *N. crassa* conidiospores were germinated for 5 h at 30°C in Vogel's minimal medium supplemented with 2% glucose (A,B) or 1% glycerol (C,D). After that time each culture was divided, harvested and germlings were resuspended in fresh medium supplemented either with 2% glucose (○,●) or 1% glycerol (△, ▲), and reincubated at 30°C (○, △) or 45°C (●, ▲). At the times represented in the abscissa aliquots of the cultures were sampled and processed for determination of trehalose (A,C), glycogen (B,D) and protein.

that gluconeogenic activity could also provide hexoses for trehalose synthesis.

The results in Fig. 3 also indicated that the accumulation of trehalose at 45°C required an exogenous source of carbon, and that the glucose produced from the degradation of glycogen under these conditions did not significantly contribute for trehalose synthesis. For instance, one can observe that glucose-germinated cells incubated at 45°C in glycerol-supplemented medium degraded an amount of glycogen roughly equivalent to 1.75 μmol of glucose/mg protein, without measurable effects on the level of trehalose (Fig. 3B), while glycerol-germinated cells incubated at 45°C accumulated trehalose with little glycogen degradation (Fig. 3D).

As shown above, by manipulating the carbon source used for germination and during the heat shock of the conidiospores, it was possible to control their concentration of trehalose and glycogen. This allowed us to examine the influence of these carbohydrates on the acquisition of thermotolerance. Results in Table I showed that higher levels of trehalose increased the thermotolerance of conidiospores, as assessed by a treatment at 55°C for 30 min. However, there is no linear

Table I

Effect of heat shock on the level of trehalose, glycogen and thermotolerance of wild-type *N. crassa* germlings incubated in glucose or glycerol

Incubation conditions[a]	Trehalose content (μmol/mg protein)	Glycogen content (μmol glucose/mg protein)	Survival fraction (10^{-5})
Glucose-germinated cells			
60 min at 30°C in glucose	0.27	2.51	1
60 min at 45°C in glucose	1.35	0.94	47
60 min at 30°C in glycerol	0.19	0.41	4
60 min at 45°C in glycerol	0.22	0.38	5
Glycerol-germinated cells			
60 min at 30°C in glucose	0.35	1.71	2
60 min at 45°C in glucose	1.99	1.29	190
60 min at 30°C in glycerol	0.51	2.22	1
60 min at 45°C in glycerol	1.19	1.71	180

[a] Wild-type conidiospores were incubated for 5 h at 30°C in 2% glycose or 1% glycerol-supplemented medium, as indicated. After that, the germlings were harvested, resuspended in fresh medium supplemented with either glucose or glycerol as indicated, incubated for an additional one hour period at 30°C or 45°C and then incubated at 55°C for 30 min. Trehalose and glycogen content were determined after the 45°C treatment and thermotolerance after the incubation at 55°C. Other details in section 2

correlation between trehalose content and survival fraction, suggesting that the acquisition of thermotolerance involved other processes than the presence of trehalose. Data in Table I also indicated that the level of glycogen in the cells had no correlation with their ability to survive during exposure at 55°C.

These results pointed out a rather striking parallelism in the heat shock effects on trehalose and glycogen metabolism and on the induction of thermotolerance of *N. crassa* and *S. cerevisiae*. Nevertheless, the molecular and regulatory basis of at least trehalose metabolism actually are different in the two organisms [2,21]. In a recent report, De Virgilio et al. [7] describe very similar responses to heat shock in the fission yeast *Schizosaccharomyces pombe*. This organism also accumulates trehalose during sporulation and possesses, like *N. crassa* [2] a trehalase with acid pH optimum, specifically associated with the cell wall [22]. Both in *S. cerevisiae* and *S. pombe*, heat shock increases the activity of trehalase and trehalose, 6-phosphate synthase [4,7]. Thus, our next steps will be to investigate the consequences of the temperature shifts on the activity of the enzymes of trehalose and glycogen metabolism in *N. crassa*.

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