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Aerobic heat shock activates trehalose synthesis in embryos of *Artemia franciscana*

James S. Clegg and Susan A. Jackson

University of California, Davis Bodega Marine Laboratory, Bodega Bay, CA 94923, USA

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Encysted embryos (cysts) of the brine shrimp, *Artemia franciscana*, contain large amounts of trehalose which they use as a major substrate for energy metabolism and biosynthesis for development under aerobic conditions at 25°C. When cysts are placed at 42°C (heat shock) these pathways stop, and the cysts re-synthesize the trehalose that was utilized during the previous incubation at 25°C. Glycogen and glycerol, produced from trehalose at 25°C, appear to be substrates for trehalose synthesis during heat shock. Anoxia prevents trehalose synthesis in cysts undergoing heat shock. These results are consistent with the view that trehalose may play a protective role in cells exposed to heat shock, and other environmental insults, in addition to being a storage form of energy and organic carbon for development.

Trehalose; Heat shock; Glycogen; Glycerol; *Artemia franciscana*

1. INTRODUCTION

The resistant stages of a wide variety of organisms contain large concentrations of the disaccharide trehalose (1-α-D-glucopyranosyl-1,1-D-α-glucopyranoside) [1,2]. In some cases this sugar is a storage form of carbohydrate, and it also appears to protect some cells against the damage of heat shock, desiccation, and freezing (reviewed in [3]), although in yeast cells there is evidence that the situation is not so simple [4]. Nevertheless, a substantial body of data indicates that the protective effect of trehalose involves a complex interaction with the hydrated surfaces of membranes, proteins and, perhaps, other macromolecules [3,5]. Although most research on trehalose and its metabolism has been done on microorganisms (see [2, 6–11]) and various in vitro preparations [3,12], the encysted embryos (cysts) of the brine shrimp, *Artemia franciscana*, have also proved to be a useful model system in which to study the roles of trehalose [5]. The early development of this organism results in the deposition of about 15% of their dry weight as trehalose when they enter diapause (dormancy) as encysted gastrula embryos [13]. When diapause is broken [14] trehalose metabolism is initiated, some of it undergoing complete oxidation and the remainder serving as a substrate for glycogen and glycerol synthesis [15]. Although these pathways are not reversible at 25°C we will show here that an aerobic heat shock of 42°C results in a rapid re-synthesis of trehalose and a shutdown in its oxidation to CO_2 and H_2O, and conversion to glycerol and glycogen.

Correspondence address: J.S. Clegg, University of California, Davis Bodega Marine Laboratory, Bodega Bay, CA 94923, USA. Fax: (1) (707) 875-2089.

2. MATERIALS AND METHODS

Cysts from the south San Francisco Bay, obtained in 'vacuum-packed' cans (San Francisco Bay Brand, Hayward, CA) were processed and stored as described previously [16]. For aerobic incubation the cysts were hydrated in seawater (SW) at 2°C and then transferred to 25°C in a shaking incubator (50 rpm) for 4 h. They were harvested by rapid filtration, rinsed with ice-cold distilled water, and samples taken for analysis (see later) and transfer to 42°C (± 0.1°). Pre-heated (42°C) air was continuously bubbled through the medium to maintain aerobic conditions. After incubation, known wet weights of cysts (about 80 mg) were transferred to glass homogenizers, and to tared aluminum foil cups for dry weight measurements (103°C, 24 h). For heat shock under anoxic conditions, SW was degassed with ultrapure helium (99.995% from Liquid Carbonic, Chicago, IL) for 4 h at 42°C. After a 4 h aerobic incubation at 25°C the cysts were harvested and transferred to 42°C anoxic SW These tubes were continuously gassed with helium during incubation at 42°C. Anoxic incubation at 25°C was carried out in sealed glass vials as described in detail previously [17].

For analysis cysts were homogenized at 0°C in 1.0 ml of 72% ethanol and the homogenate transferred to centrifuge tubes with another 1.0 ml of ethanol. After centrifugation (2°C, 10 min at 1,600 × g) the ethanol-soluble fraction (ESC) was removed for analysis of total carbohydrate using a phenol–sulfuric acid assay [18] and for trehalose and glycerol using HPLC [17]: Aminex HPX-87H cation-exchange column (Bio-Rad), mobile phase 0.01 N H_2SO_4, flow rate 0.6 ml/min, 41°C. A Knauer differential refractometer was used to detect and quantify glycerol and trehalose by peak integration. Glycogen in the ethanol-soluble pellet was extracted using a KOH method and quantified colorimetrically [19].

Hatching (viability) assays were carried out in sealed plastic depression plates using aerobic SW at 22–23°C in constant light. The percentage of larvae hatching from cysts was determined after 5 days of incubation.

3. RESULTS

Fig. 1 shows the concentrations of glycogen and total ethanol-soluble carbohydrates (ESC) in cysts incubated at 25°C for 4 h, followed by incubation at 42°C under aerobic conditions or under anoxic conditions at 25°C. As expected, ESC (most of which will be shown to be trehalose) decreased and glycogen increased during the 25°C incubation. When these cysts were transferred to 42°C, the original level of ESC was restored by 24 h, and maintained at that level for an additional 24 h. Glycogen decreased at 42°C, but the amount of this decrease could account for only about half of the increase in ESC

When cysts were placed under anoxic conditions at 25°C after 4 h of aerobic incubation, glycogen decreased, but no increase in ESC occurred, even after a year of incubation (Fig. 1).

To examine the aerobic heat shock response further we also measured trehalose and glycerol (another major cyst component) during incubation at 42°C following the usual 4 h 25°C incubation (Fig. 2). This representative study shows that trehalose synthesis occurs rather quickly, and that the final amount of trehalose synthesized is about equal to the summed decrease in glycerol and glycogen taking place during the 24 h heat shock. The final level of ESC (28 h) shows an increase larger than that present when heat shock began, although that does not always occur (Fig. 1). The nature of the non-trehalose ESC is not known; however, it is not free glucose, which could not be detected by HPLC (limit of detection < 0.2 μg/mg dry cysts). Since cysts, when fully hydrated, contain about 1.4 mg H_2O/mg dry weight [16] the global concentration of glucose is estimated at less than 0.7 mM.

Table I provides a quantitative summary of changes

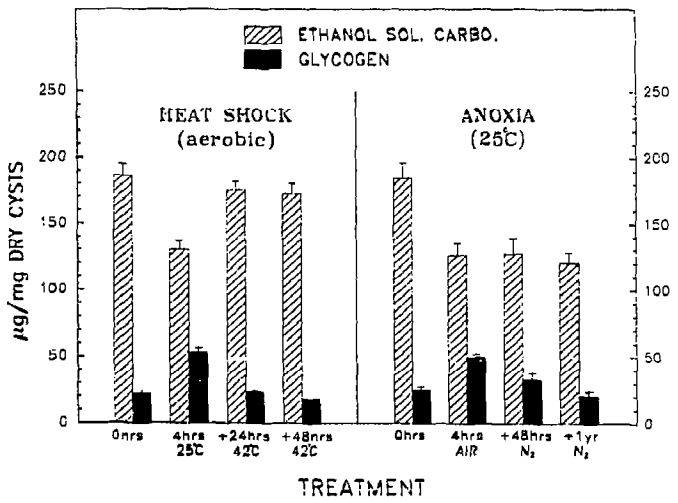

Fig. 1. Concentrations of total ethanol-soluble carbohydrates and glycogen in *Artemia* cysts before and after aerobic incubation at 25°C followed by aerobic heat shock (42°C) or anoxia (25°C). Bars represent one standard deviation (n=3).

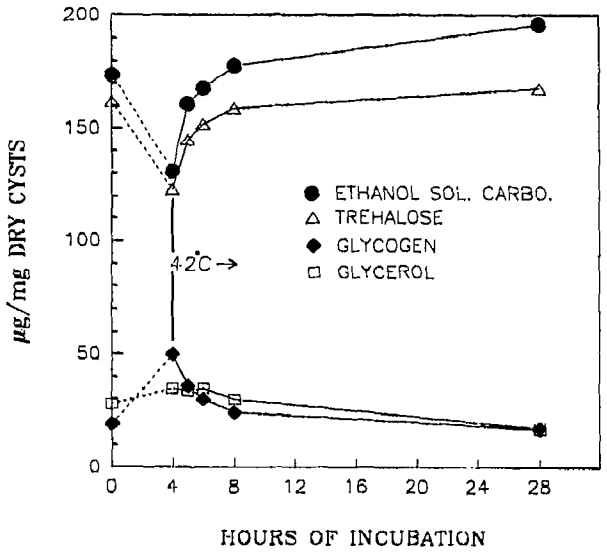

Fig. 2. Concentrations of various compounds in *Artemia* cysts after 4 h of aerobic incubation (dashed lines) followed by exposure to 42°C (solid lines).

in trehalose, glycerol and glycogen after a 24 h heat shock. The average increase in trehalose (44 μg) is accompanied by decreases in glycerol (16 μg) and glycogen (30 μg). Trehalose after heat shock was restored to its original level (0 time) but net decreases in glycerol and glycogen took place during this time.

Since anoxia at 25°C did not result in trehalose (ESC) synthesis (Fig. 1) we asked whether heat shock under anoxic conditions would prevent that synthesis, and that is the result obtained (Table II). Thus, heat shock-induced trehalose synthesis is an aerobic process.

Hatching assays have been carried out on these preparations. Controls (unincubated cysts) exhibit a hatch level of 90% (±3, standard deviation for 4 batches of at least 200 cysts) while those undergoing 42°C for 24 and 48 h produce 83% ± 4 S.D., and 86% ± 3 S.D., respectively.

4. DISCUSSION

To our knowledge no previous evidence has been obtained for the synthesis of trehalose in post-diapause

Table I

Effect of aerobic heat shock on the levels of various compounds in *Artemia* cysts after pre-incubation at 25°C for 4 h

Incubation conditions	μg/mg dry wt. cysts ± S.E.M. (n=4)[a]		
	Trehalose	Glycerol	Glycogen
zero time[b]	169 ± 4	26 ± 2	20 ± 1
+ 4 h 25°C	127 ± 3	33 ± 1	46 ± 2
+ 24 h 42°C	171 ± 4	18 ± 1	16 ± 1

[a] S.E.M. is the standard error of the mean for 4 replicates (n)
[b] Refers to cysts prior to incubation

Artemia embryos under any condition. Therefore, the results presented here establish that the enzymes needed for trehalose synthesis are either retained after diapause in an inactive state or, less likely in our opinion, that they are synthesized rapidly upon exposure to 42°C.

The general pattern of heat shock-induced trehalose synthesis has previously been observed in fungi where it seems to be a widespread phenomenon (for reviews see [7–10]). Although it has been suggested that trehalose in fungi may protect cells against heat shock [7,8] no direct evidence exists at present.

The Crowes and their colleagues have produced a substantial body of evidence that trehalose protects membrane structure and function against desiccation damage (reviewed in [1]). Whether or not trehalose can mitigate membrane damage and the denaturation of proteins by high temperature remains to be evaluated.

Although glycogen and glycerol are likely candidates for trehalose synthesis during heat shock the impermeability of these cysts [14,19] precludes appropriate studies to test this using radioactive precursors. Whatever the pathways involved, the process is dependent on aerobic conditions since anoxia prevents heat shock-induced trehalose synthesis (Table II).

The low global level of free glucose in these cysts, under all conditions of incubation, has interesting metabolic implications. Since trehalose breakdown in *Artemia* cysts appears to involve its hydrolysis by trehalases (see [14,20,21]) one might expect to observe significant concentrations of free glucose. Therefore, it appears that the glucose resulting from trehalose hydrolysis may be limited to microcompartments. It is also possible that the enzymes of the initial steps of trehalose metabolism are highly organized, possibly involving glucose channeling.

Comment should be made about previous work on heat shock in these cysts (see [22,23]), from which we selected the temperature used in the present paper. The synthesis of several heat shock proteins (hsp) is constitutive in this system, but enhanced by heat shock [23]. One of these appears to be a protein called 'artemin' (see [24,25]). Interesting features of this protein are its presence in very large amounts (about 13% of total 'soluble' protein), its disappearance during development of the encysted embryo into a free-swimming larva and its similarity to vertebrate ferritin which has recently been shown to be a heat shock protein in avian reticulocytes [26].

Table II

Effect of anoxic heat shock on the levels of various compounds in *Artemia* cysts after pre-incubation at 25°C for 4 h

Incubation conditions	μg/mg dry wt. cysts ± S.E.M. (n=3)[a]		
	Trehalose	Glycerol	Glycogen
zero time[b]	171 ± 5	27 ± 3	21 ± 2
+ 4 h 25°C (aerobic)	132 ± 2	37 ± 1	56 ± 3
+ 24 h 42°C (anoxic)	130 ± 2	35 ± 1	45 ± 1

[a] S.E.M. is the standard error of the mean for 3 replicates (n)
[b] Refers to cysts prior to incubation

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