The Journal of Experimental Biology 207, 3125-3129
Published by The Company of Biologists 2004
doi:10.1242/jeb.01133

Review

Role of trehalose phosphate synthase and trehalose during hypoxia: from flies to mammals

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Accepted 9 June 2004

Summary

Trehalose is a nonreducing disaccharide in which the two glucose units are linked in an α,α-1,1-glycosidic linkage. The best known and most widely distributed pathway of trehalose synthesis involves the transfer of glucose from UDP-glucose to glucose 6-phosphate to form trehalose-6-phosphate and UDP *via* the trehalose-6-phosphate synthase (TPS1). Trehalose-6-phosphate phosphatase (TPS2) then converts trehalose-6-phosphate to free trehalose. This sugar is present in a wide variety of organisms, including bacteria, yeast, fungi, insects, invertebrates and plants, and because of its particular physical features, trehalose is able to protect the integrity of cells against a variety of environmental stresses such as desiccation, dehydration, heat, cold and oxidation. Our current studies described here indicate that trehalose protects *Drosophila* and mammalian cells from hypoxic and anoxic injury. The mechanism of this protection is probably related to a decrease in protein denaturation through protein–trehalose interactions.

Key words: trehalose, glucose, hypoxia, anoxia, trehalose phosphate synthase.

Introduction

One of the devastating human conditions relates to O_2 insufficiency to organs, especially to the brain. Although many clinical trials have been launched in the past decade or two to treat stroke patients, not a single one has proven effective and we still have no effective treatment for ischemic brain diseases and stroke. This contrasts with a plethora of research on mechanisms of injury, damage and mechanisms of survival after O_2 insufficiency. We and others have worked on many mechanisms, including ionic homeostasis, the role of cytoskeletal proteins, chaperone proteins, mechanisms of cell death, gene regulation and cell–cell communication.

In this review, we capitalize on some of our most recent work on an invertebrate, *Drosophila melanogaster*, and focus on the role of some chaperones in hypoxic injury or survival. We will review how trehalose, a disaccharide, works in general, its role in *Drosophila* and in mammalian cells.

Trehalose: molecular properties

Distribution in nature

Trehalose is a disaccharide in which two glucose molecules are linked together in a α,α-1,1-glycosidic linkage. It is a non-reducing sugar that is not easily hydrolyzed by acid, and the glycosidic bond is not cleaved by glucosidase. Physical properties that make trehalose unique are its high degree of optical rotation ($[\alpha]_D^{20}$+178°) and its melting behavior. Trehalose first melts at 97°C, additional heat drives off the water of crystallization until the material resolidifies at 130°C, and then the anhydrous trehalose melts at 203°C. The combination of the molecular structure and physico-chemical properties of trehalose result in a very stable disaccharide (Birch, 1963; Elbein, 1974). It is also a unique disaccharide in that it doesn't have a free aldehyde group (or hemiacetal hydroxide) and therefore doesn't react with free amino groups in a non-enzymic glycation reaction.

This naturally occurring disaccharide is widespread throughout the biological world. Elbein summarized the distribution of 1,1-trehalose in over 80 species representing plants, algae, fungi, yeasts, bacteria, insects and other invertebrates (Elbein, 1974). From the wide variety of species that have been shown to contain trehalose, it seems likely that trehalose may be present in many other organisms.

In the animal kingdom, trehalose was first reported in insects, where it is present in the hemolymph and also in larvae or pupae. In the adult insect, the levels of trehalose fall rapidly during certain energy-requiring activities, such as flight, indicating a role for this disaccharide as a source of glucose for energy (Elbein et al., 2003). From our studies we found that the metabolic pool of trehalose was active in *Drosophila* (discussed later). Trehalose is not found in higher species (mammals), even though trehalase has been found in

significant amounts in the small intestine and other organs of various species (Richards et al., 2002).

Synthesis and degradation

The best studied pathway for the biosynthesis of α,α-1,1-trehalose is that involving the enzyme trehalose-phosphate synthase (TPS1), which catalyzes the transfer of glucose from UDP-glucose to glucose-6-phosphate to produce trehalose-6-phosphate plus UDP; then trehalose-6-phosphate is hydrolyzed to trehalose by trehalose-6-phosphate phosphatase (TPS2). This reaction was first described in yeast (Cabib and Leloir, 1958) and has since been demonstrated in numerous organisms, including insects (Murphy and Wyatt, 1965) and plants (Eastmond et al., 2002; Vogel et al., 2001). Trehalase is an enzyme that specificly hydrolyzes trehalose, yielding glucose.

Trehalose levels may vary greatly in certain cells depending on the stage of growth, the nutritional state of the organism, and the environmental conditions. In insects, trehalose is a major sugar in the hemolymph and thorax muscles and is consumed during flight. Trehalose is also an important component in fungal spores, where trehalose hydrolysis is a major event during germination and can serve as a source of carbon (Elbein et al., 2003). In mycobacteria, trehalose can be incorporated into glycolipids and therefore acts as a structural component (Elbein and Mitchell, 1973).

Function of trehalose

Protein integrity

Modern medicine requires preservation of tissue and blood cells for transplantations or transfusions. Trehalose has attracted attention for this purpose. Zhang et al. (2003) showed that 5% trehalose with 10% dimethylsulfoxide (DMSO) increased the recovery of committed progenitor cells (CFU-GM, CFU/BFU-E), early progenitors (CFU-GEMM), and long-term culture-initiating cells (LTC-IC) by over 7.25% (mean), 11.9%, 19.2% and 12.9%, respectively, when compared to those in 10% DMSO alone (Zhang et al., 2003). Crowe and coworkers found that trehalose can be taken up by human platelets at 37°C with loading efficiencies of 50% or greater, due to fluid-phase endocytosis and other unknown mechanisms (Wolkers et al., 2001). Trehalose-loaded platelets were successfully freeze-dried, with excellent recovery of intact platelets. Rehydration from the vapor phase led to a survival rate of 85%, with a shelf life of at least 6 months at room temperature. The response of these platelets to agonists such as thrombin (1 U ml^{-1}), collagen (2 mg ml^{-1}), ADP (20 mmol l^{-1}) and ristocetin (1.6 mg ml^{-1}) was almost identical to that of fresh, control platelets. Analysis by infrared spectroscopy demonstrated that the membrane and protein components of trehalose-loaded platelets after freeze-drying, prehydration and rehydration were remarkably similar to those of fresh platelets (Wolkers et al., 2001).

Heat, dehydration and oxidant stress

Organisms have evolved various mechanisms for adaptation to adverse environmental conditions such as lack of water (dehydration) and high temperature. For example, a chironomid, *Polypedilum vanderplanki* Hint., is the largest multicellular animal known to tolerate almost complete dehydration without damage. Cryptobiotic larvae show extremely high thermal tolerance from –270°C to +106°C and can recover soon after prolonged dehydration of up to 17 years (Hinton, 1960).

One of the mechanisms for the tolerance of *Polypedilum vanderplanki* to extreme conditions is that larvae can rapidly accumulate a large amount of trehalose (18% of dry body mass) (Watanabe et al., 2002). Soto et al. (1999) examined the effects of trehalose-6-phosphate (trehalose-6P) synthase overexpression on resistance to several stresses in cells of *S. pombe* transformed with a plasmid bearing the *tps1* gene, which codes for trehalose-6-phosphate synthase, under the control of the strong thiamine-repressible promoter. Upon induction of trehalose-6-phosphate synthase, the elevated levels of intracellular trehalose correlated not only with increased tolerance to heat shock but also with resistance to other stresses such as freezing and thawing, dehydration and osmostress, and toxic levels of ethanol (Soto et al., 1999).

Introduction of trehalose into plant and mammalian cells using transgenic techniques increases resistance to drought and desiccation. For example, Garg et al. (2002) overexpress *Escherichia coli* trehalose biosynthetic genes (*otsA* and *otsB*) as a fusion gene under the control of stress-dependent promoters in rice. The transgenic rice plants accumulated trehalose at levels 3–10 times that of the nontransgenic controls. Compared with nontransgenic rice, several independent transgenic lines exhibited sustained plant growth, less photo-oxidative damage, and more favorable mineral balance under salt, drought and low-temperature stress conditions (Garg et al., 2002). Similar results were obtained with human fibroblasts that had the *otsA* and *otsB* genes inserted and expressed. These cells could be maintained in the dry state for up to 5 days, as compared with control cells that were very sensitive to drying (Guo et al., 2000). Trehalose at 50, 100 and 200 mmol l^{-1} protected corneal epithelial cells in culture from death by desiccation (Matsuo, 2001).

Tissue injury due to oxidant species is present in many clinical settings, such as hypoxic pulmonary hypertension, ARDS (acute respiratory distress syndrome) and coronary heart disease. Though there is no evidence that trehalose can reduce oxidant injury in mammalian cells, a large body of evidence has been collected in *Candida albicans* and *S. cerevisiae*, indicating that trehalose could be a promising free radical scavenger. Growing *Candida albicans* cells from trehalose-deficient mutant were extremely sensitive to severe oxidative stress exposure (H_2O_2), while in wild-type cells H_2O_2 exposure induced intracellular accumulation of trehalose and a higher survival rate after the same exposure. Exposure of *Saccharomyces cerevisiae* to a mild heat shock (38°C) or to

a proteasome inhibitor (MG132) induced trehalose accumulation and markedly increased the viability of the cells upon exposure to a free radical-generating system (H_2O_2/iron). When cells were returned to normal growth temperature (28°C) or MG132 was removed from the medium, the trehalose content and resistance to oxygen radicals decreased rapidly. Providing trehalose exogenously enhanced the resistance of mutant cells to H_2O_2, and trehalose accumulation was found to reduce oxidative damage to amino acids in cellular proteins (Benaroudj et al., 2001).

Tps1 *and trehalose synthesis in* Drosophila

We used yeast *tps1* cDNA to blast the *Drosophila* database, and found a gene with a 2427 bp open reading frame; this *Drosophila* gene was found to be 30% similar to yeast *tps1*. *Drosophila* cDNA library screening was performed using part of the sequence as a probe. We cloned this gene, and its amino acid sequence was compared to yeast TPS1 and TPS2. *Drosophila* TPS1 shows 29.7% identity to *S. cerevisiae* TPS1 and 17.4% and 22.5% identity to *S. cerevisiae* TPS2 and *S. lepidophylla* TPS1, respectively. Our experiment of overexpression of the gene in *Drosophila* and mammalian cells confirmed that its function is in synthesis of trehalose using UDP-glucose and glucose-6-phosphate as substrate (Chen et al., 2002, 2003).

Overnight-fasted flies were exposed to a solution of [1–13C]glucose for 2 h, and fly heads were subjected to measurement of 12C- and 13C-labeled trehalose using NMR. We found that 40.4% of the total trehalose was labeled at 13C, confirming that the trehalose pool is metabolically active (Chen et al., 2002).

Trehalose protects Drosophila *and mammalian cells from hypoxic and anoxic injury*

The ability of organisms to sustain O_2 deprivation is limited. Irreversible injury may occur to mammalian tissues within 5–10 min of severe hypoxia or ischemia. However, *Drosophila* can tolerate a complete N_2 atmosphere for up to 4 h, after which they totally recover. One of the mechanisms that contributes to the survival of *Drosophila* is the profound decline in metabolic rate during periods of low environmental O_2 levels (Haddad et al., 1997; Haddad and Ma, 2001). Trehalose is present in flies at a concentration of 120 μg 30 mg^{-1} whole fly tissue and is metabolically active (Chen et al., 2002). We investigated whether trehalose plays an important role in protecting flies against anoxic stress. We first cloned *tps1* (the gene for trehalose-6-phosphate synthase, which synthesizes trehalose), and examined the effect of *tps1* overexpression or mutation on the resistance of *Drosophila* to anoxia. Upon induction of *tps1*, trehalose levels increased, and this was associated with increased tolerance to anoxia (Fig. 1). Furthermore, *in vitro* experiments showed that trehalose reduced protein aggregation (such as Na^+/K^+ATPase) caused by anoxia (Fig. 2). To determine whether trehalose can protect against anoxic injury in mammalian cells, we transfected the *Drosophila tps1* gene (*dtps1*) into human HEK-293 cells using

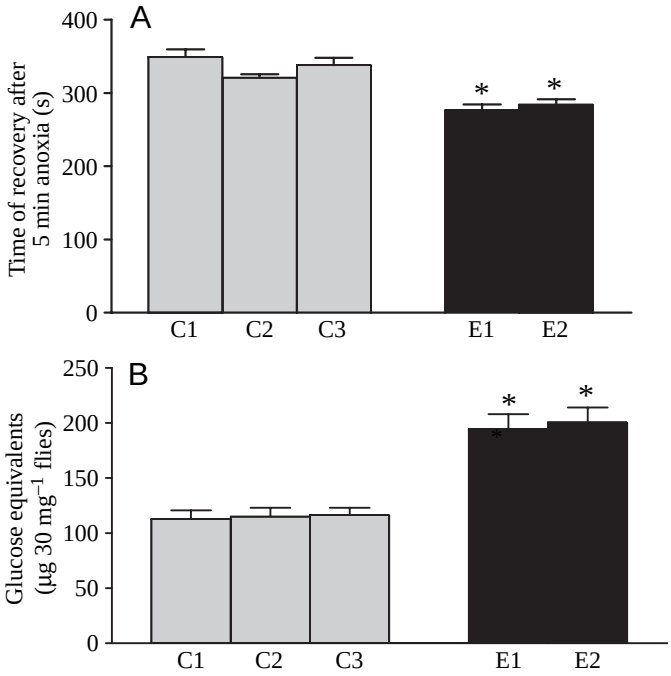

Fig. 1. Overexpression of *tps1* in adult flies increases trehalose level and increases tolerance to anoxia. (A) Flies overexpressing *tps1* (E1 and E2) have a shorter recovery time than controls (C1, C2 and C3) after exposure to 5 min anoxia. **P*<0.005 as compared with controls. (B) Flies overexpressing *tps1* contain more trehalose than controls. **P*<0.005 as compared with controls.

the recombinant plasmid pcDNA3.1(–)-*dtps1* and obtained more than 20 stable cell strains. Glucose starvation in culture showed that HEK-293 cells transfected with pcDNA3.1(–)-*dtps1* (HEK-*dtps1*) do not metabolize intracellular trehalose and, interestingly, these cells accumulated intracellular trehalose during hypoxic exposure. In contrast to HEK-293 cells transfected with pcDNA3.1(–) (HEK-v), cells with trehalose were more resistant to low oxygen stress (1% O_2). To elucidate how trehalose protects cells from anoxic injury, we assayed protein solubility and the amount of ubiquitinated proteins. There were three times more insoluble protein in HEK-v cells than in HEK-*dtps1* cells after 3 days of exposure to low O_2. The amount of Na^+/K^+ATPase present in the insoluble proteins dramatically increased in HEK-v cells after 2 and 3 days of exposure, whereas there was no significant change in HEK-*dtps1* cells. Ubiquitinated proteins increased dramatically in HEK-v cells after 2 and 3 days of exposure but not in HEK-*dtps1* cells over the same period (Fig. 3). Our results indicate that increased trehalose in mammalian cells following transfection by the *Drosophila tps1* gene protects cells from hypoxic injury. The mechanism of this protection is probably related to a decrease in protein denaturation through protein–trehalose interactions (Chen et al., 2002, 2003).

Mechanisms of action of trehalose

Different proteins are expected to interact with cosolvent molecules in varied ways depending on their physico–chemical

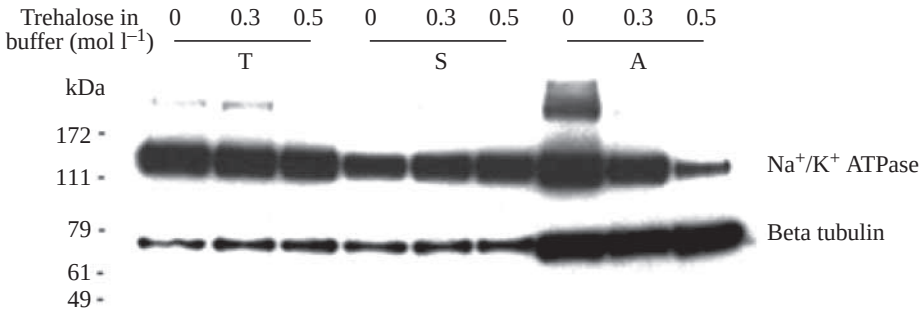

Fig. 2. Western blot of total (T), soluble (S), and aggregated (A) proteins prepared with the addition of 0, 0.3, 0.5 mol l^{-1} trehalose in extraction buffer. Flies were subjected to 4 h of anoxia and 5 μg of aggregated proteins were prepared by adding 0, 0.3, 0.5 mol l^{-1} trehalose. Although the same amount of Na^+/K^+ATPase seemed to be present in total proteins, much less Na^+/K^+ATPase was present in the aggregated proteins with 0.5 mol l^{-1} trehalose as compared with that lacking trehalose. Note that the beta-tubulin concentration did not change in each of T, S or A proteins as a function of trehalose.

properties. However, trehalose has been observed to provide protection to different proteins to various extents and the efficacy of protection depends on the nature of the protein. To understand the mechanism of action of trehalose in detail,

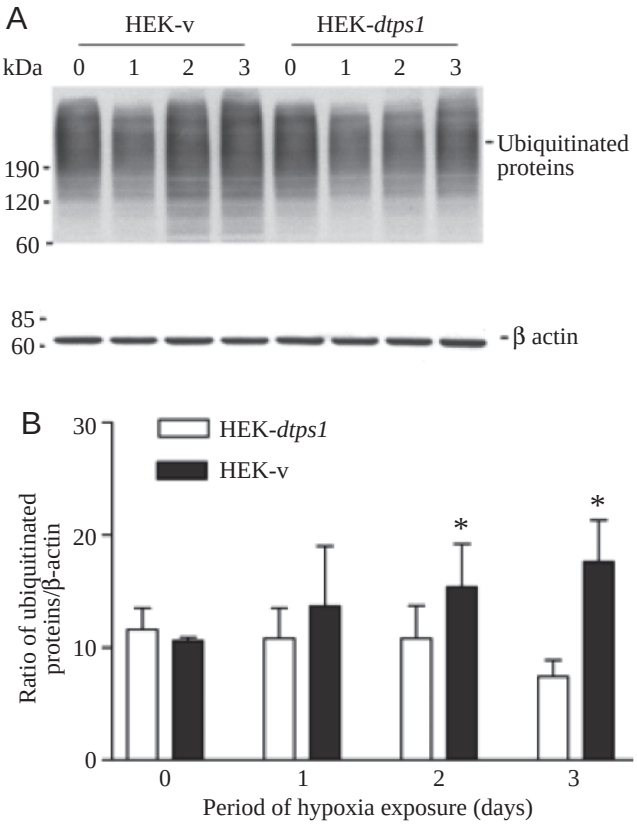

Fig. 3. Intracellular trehalose reduces ubiquitinated proteins during hypoxia. (A) Western signals of ubiquitinated proteins and β-actin. (B) Relative amount of ubiquitinated proteins per same amount of total proteins after hypoxic treatment for 0, 1, 2 and 3 days in HEK-v and HEK-*dtps1* cells. This figure shows that there was almost no difference in the ratio of ubiquitinated proteins/β-actin between HEK-v and HEK-*dtps1* cells at days 0 and 1 of hypoxia exposure. However, as exposure time increased, there was a significant difference on days 2 and 3. **P*<0.05 compared to HEK-*dtps1* cells.

Kaushik and Bhat (2003) conducted a thorough investigation of its effect on the thermal stability in aqueous solutions of five well-characterized proteins differing in their various physico-chemical properties. Among them, RNase A has been used as a model enzyme to investigate the effect of trehalose on the retention of enzymatic activity upon incubation at high temperatures. Trehalose was observed to raise the stability temperature of RNase A by as much as 18°C and Gibbs free energy by 4.8 kcal mol^{-1}. There is a decrease in the heat capacity of protein denaturation in trehalose solutions for all the studied proteins. An increase in free energy and a decrease in protein denaturation values for all the proteins point toward a general mechanism of stabilization due to the elevation and broadening of the stability curve (free energy *versus* temperature). They further show that an increase in the stability of proteins in the presence of trehalose depends upon the length of the polypeptide chain. Their pH dependence data suggest that even though the charge status of a protein contributes significantly, trehalose can be expected to work as a universal stabilizer of protein conformation due to its exceptional effect on the structure and properties of solvent water compared with other sugars and polyols (Kaushik and Bhat, 2003). The continued presence of trehalose, however, can interfere with refolding, demonstrating the importance of its rapid hydrolysis following heat shock (Singer and Lindquist, 1998).

During the freeze-drying process, or on subsequent storage in the dry state, the protein conformation may be changed, exposing highly reactive sites that are prone to physical and chemical changes over prolonged periods (Service, 1997). This could lead to protein degradation, and to a loss in biological activity. Carpenter and coworkers showed that sugars form hydrogen bonds with the protein in a glassy matrix to maintain the protein's native conformation with reduced mobility during lyophilization and storage (Allison et al., 1999). These investigators have also shown that molecular compatibility between components is an important factor for determination of their propensity to phase separate and crystallize (Izutsu et al., 1996). The amorphous nature of trehalose is more 'compatible' with the protein than the crystalline nature of

lactose, which could result in phase separation of the protein and the sugar. Lam et al. (2002), studied protein mobility in lyophilized protein–sugar powders using solid-state NMR, and their results indicated that trehalose was 'bound' to lysozyme while the lactose phase separated during lyophilization and storage, which makes trehalose a better protectant than lactose (Lam et al., 2002).

In summary, trehalose is an important protectant of protein integrity and seems to be important during stress. It is possible that some of the mechanisms that are important for recovery from stress are shared not only between organisms but also between various types of stresses. Trehalose is an example of how a disaccharide molecule can enhance protein integrity and limit protein degradation not only under heat stress and oxidant injury but also in anoxia. This is also an example of a mechanism that is helpful not only in *Drosophila* but in mammalian cells.

This work was supported by National Institutes of Health Grants P01-HD32573-09 and R01-NS37754 (to G.G.H.).

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