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REVIEW

New insights on trehalose: a multifunctional molecule

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Trehalose is a nonreducing disaccharide in which the two glucose units are linked in an α,α-1,1-glycosidic linkage. This sugar is present in a wide variety of organisms, including bacteria, yeast, fungi, insects, invertebrates, and lower and higher plants, where it may serve as a source of energy and carbon. In yeast and plants, it may also serve as a signaling molecule to direct or control certain metabolic pathways or even to affect growth. In addition, it has been shown that trehalose can protect proteins and cellular membranes from inactivation or denaturation caused by a variety of stress conditions, including desiccation, dehydration, heat, cold, and oxidation. Finally, in mycobacteria and corynebacteria, trehalose is an integral component of various glycolipids that are important cell wall structures.

There are now at least three different pathways described for the biosynthesis of trehalose. The best known and most widely distributed pathway involves the transfer of glucose from UDP-glucose (or GDP-glucose in some cases) to glucose 6-phosphate to form trehalose-6-phosphate and UDP. This reaction is catalyzed by the trehalose-P synthase (TPS here, or OtsA in *Escherichia coli*). Organisms that use this pathway usually also have a trehalose-P phosphatase (TPP here, or OtsB in *E. coli)* that converts the trehalose-P to free trehalose. A second pathway that has been reported in a few unusual bacteria involves the intramolecular rearrangement of maltose (glucosyl-α1,4-glucopyranoside) to convert the 1,4-linkage to the 1,1-bond of trehalose. This reaction is catalyzed by the enzyme called trehalose synthase and gives rise to free trehalose as the initial product. A third pathway involves several different enzymes, the first of which rearranges the glucose at the reducing end of a glycogen chain to convert the α1,4-linkage to an α,α1,1-bond. A second enzyme then releases the trehalose disaccharide from the reducing end of the glycogen molecule. Finally, in mushrooms there is a trehalose phosphorylase that catalyzes the phosphorolysis of trehalose to produce glucose-1-phosphate and glucose. This reaction is reversible *in vitro* and could theoretically give rise to trehalose from glucose-1-P and glucose.

Another important enzyme in trehalose metabolism is trehalase (T), which may be involved in energy metabolism and also have a regulatory role in controlling the levels of trehalose in cells. This enzyme may be important in lowering trehalose concentrations once the stress is alleviated. Recent studies in yeast indicate that the enzymes involved in trehalose synthesis (TPS, TPP) exist together in a complex that is highly regulated at the activity level as well as at the genetic level.

Key words: biosynthesis of trehalose/energy/glycolipids of trehalose/membrane stabilizer/metabolism of trehalose

Introduction

In 1974, the current view on the role of trehalose was that it served as a storehouse of glucose for energy and/or for synthesis of cellular components (Elbein, 1974). Since that time, our knowledge on the various functions of this simple disaccharide has greatly expanded; it is now clear that trehalose is much more than simply a storage compound. Certainly it can and does function in that capacity in some organisms, but in others it has a structural or transport role (Takayama and Armstrong, 1976), whereas in still others it may be involved in signaling or regulation, or functions to protect membranes and proteins against the adverse effects of stresses, such as heat, cold, desiccation, and anoxia (Crowe *et al.*, 1984). This review discusses our current understanding of the biosynthesis and turnover of trehalose, its various functions in cells, and its significance in homeostasis of those organisms that produce it.

Structure and distribution of trehalose

Trehalose is a nonreducing disaccharide in which two glucose molecules are linked together in a 1,1-glycosidic linkage. Although there are three possible anomers of trehalose, that is, α,β-1,1-, β,β-1,1-, and α,α1,1-, only the α,α-trehalose (Figure 1) has been isolated from and biosynthesized in living organisms. This naturally occurring disaccharide is widespread throughout the biological world. Thus the presence of trehalose in lower orders of the plant kingdom has been known for many years, with the first tentative report being in 1832 in ergot of rye (reviewed in Elbein, 1974). Trehalose is quite common in yeast and fungi where it occurs in spores, fruiting bodies, and vegetative cells (Trevelyan and Harrison, 1956; Nwaka and Holzer, 1998). For example, the spores and macrocysts of *Dictyostelium mucoroides* have been reported to contain as much as 7% trehalose on a dry-weight basis (Clegg and Filosa, 1961),

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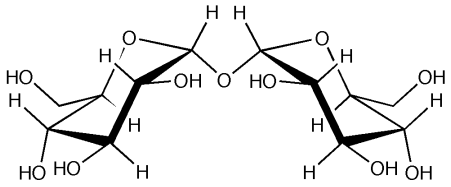

Fig. 1. Structure of the naturally occurring isomer of trehalose, α,α1,1-trehalose.

and the ascospores of *Neurospora tetrasperma* have as much as 10% trehalose (Sussman and Lingappa, 1959). When these spores germinate, the trehalose rapidly disappears suggesting that this sugar is stored as a source of carbon, and/or energy. Trehalose is also present in high concentrations in baker's (Elander and Myrback, 1949) and brewer's (Stewart *et al*., 1950) yeast; in these organisms, the levels of trehalose depend on the age of the cells, as well as on their stage of growth and their nutritional state. Many species of lichens (Lindberg, 1955) and algae (Augier, 1954) also contain this disaccharide, although in considerably lower concentrations than those found in yeast. Trehalose is probably also present in many higher plants since it has been isolated from the resurrection plant (*Selaginella lepidophylla*) (Adams *et al*., 1990) and also from *Arabidopsis thaliana* (Muller *et al*., 2001). It is apparently also a component of the wound exudates of *Fraxsinus aras* (Sabry and Atallah, 1961).

Trehalose is also found in a number of different bacteria, including *Streptomyces hygroscopicus* and other species of *Streptomyces* (Martin *et al*., 1986), various mycobacteria, including *Mycobacterium smegmatis* and *tuberculosis* (Elbein and Mitchell, 1973) and corynebacteria (Shimakata and Minatagawa, 2000). In mycobacteria and corynebacteria, this disaccharide plays a structural role as a cell wall component, but it may also serve other functions in these organisms (see later discussion). It is also present in *Escherichia coli* (Kaasen *et al*., 1994) and a number of other bacteria, such as *Rhizobium* sp. (Maruta *et al*., 1996a), *Sulfdolobus acidocaldarius* (Maruta *et al*., 1996b), *Pimelobacter* sp. R48 (Nishimoto *et al*., 1995), *Arthrobacter* sp. Q36 (Maruta *et al*., 1996c), and so on. In many of these organisms, the function of trehalose is still not clear. Several of the organisms listed appear to have rather unusual biosynthetic pathways for synthesizing trehalose, as is discussed later.

In the animal kingdom, trehalose was first reported in insects, where it is present in hemolymph (Wyatt and Kalf, 1957) and also in larvae or pupae (Fairbairn, 1958). In the adult insect, the levels of trehalose fall rapidly during certain energy-requiring activities, such as flight (Evans and Dethier, 1957), indicating a role for this disaccharide as a source of glucose for energy (see later discussion). In addition to insects, trehalose has also been identified in the eggs of the roundworm *Ascaris lumbricoides*, in which it may be present at levels as high as 8% of the dry weight (Fairbairn and Passey, 1957), and in adult roundworms and *Porrocaecum* larvae where its levels are as high as 6% of the dry weight (Kalf and Rieder, 1958). This sugar also occurs in a number of invertebrates (Friedman, 1960). This profile of the occurrence of α,α-trehalose among such diverse living organisms demonstrates its widespread distribution and suggests that it plays an important role in the biological world.

A series of trehalose oligosaccharides was isolated from the cytoplasm of *Mycobacterium smegmatis* and characterized by nuclear magnetic resonance, mass spectrometry, methylation analysis, and enzymatic digestions. These oligosaccharides all had trehalose as the base with one or two other sugars attached to it. Two trisaccharides were identified as Glcα1-4Trehalose and Glcβ1-6Trehalose, whereas three tetrasaccharides were characterized as Glcβ1-6Glcβ1-6Trehalose, Galα1-6Galα1-6Trehalose, and a trehalose with an α1-6Gal on one of its glucose residues and an α1-4Glc on the other glucose (Ohta *et al*., 2002). The function of these trehalose oligosaccharides is not known at this time, but they may be involved in stabilizing cellular structures. For example, sucrose, which is the major nonreducing disaccharide in plants, may be somewhat analogous to trehalose in terms of some of its functions (see *Function*). Thus, many plants produce higher homologs of sucrose such as raffinose (Galα1-6Sucrose) and stachyose (Galα1-6Galα1-6Sucrose); these higher sucrose oligosaccharides have been proposed to play a role in stabilizing or protecting cells against stress (Peterbauer *et al*., 2002). As will be indicated, trehalose and trehalose oligosaccharides may have similar functions.

Chemical synthesis of trehalose

α,α-Trehalose has been synthesized chemically using the ethylene oxide addition reaction between 2,3,4,5-tetra-O-acetyl-D-glucose and 3,4,6-tri-O-acetyl-1,2-anhydro-D-glucose (Lemieux and Bauer, 1954). This same series of reactions also gives rise to one of the other trehalose anomers, specifically, α,β-trehalose, also referred to as neotrehalose. Neotrehalose has also been synthesized using the Koenigs-Knorr reaction (Helferich and Weis, 1956). On the other hand, this anomer has not been isolated from any living organisms, although it was identified in koji extract (Matsuda, 1956). The other anomer of trehalose, β,β-trehalose, or isotrehalose, also has not been isolated from any living organisms, but it was found in starch hydrolysates (Sato and Aso, 1957); it also has been synthesized chemically using the Koenigs-Knorr reaction, as well as by a dehydration reaction (Bredereck *et al*., 1953). Trehalose also can be produced chemically by an acid reversion of glucose (Thompson *et al*., 1954). On the other hand, α,α-trehalose is the only anomer of trehalose that has been shown to be biosynthesized in many different types of organisms, as will be discussed.

Biosynthesis of trehalose

At least three different pathways for the biological synthesis of trehalose have been reported, and several other degradative but reversible enzymatic reactions could also be utilized (at least in principle) to produce this disaccharide. These various pathways leading to the production of trehalose-6-P or free trehalose are discussed here.

Enzymatic formation of trehalose-phosphate

The most widely reported and best studied pathway for the biosynthesis of α,α1,1-trehalose is that involving the enzyme trehalose-phosphate synthase (OtsA in *E. coli*;

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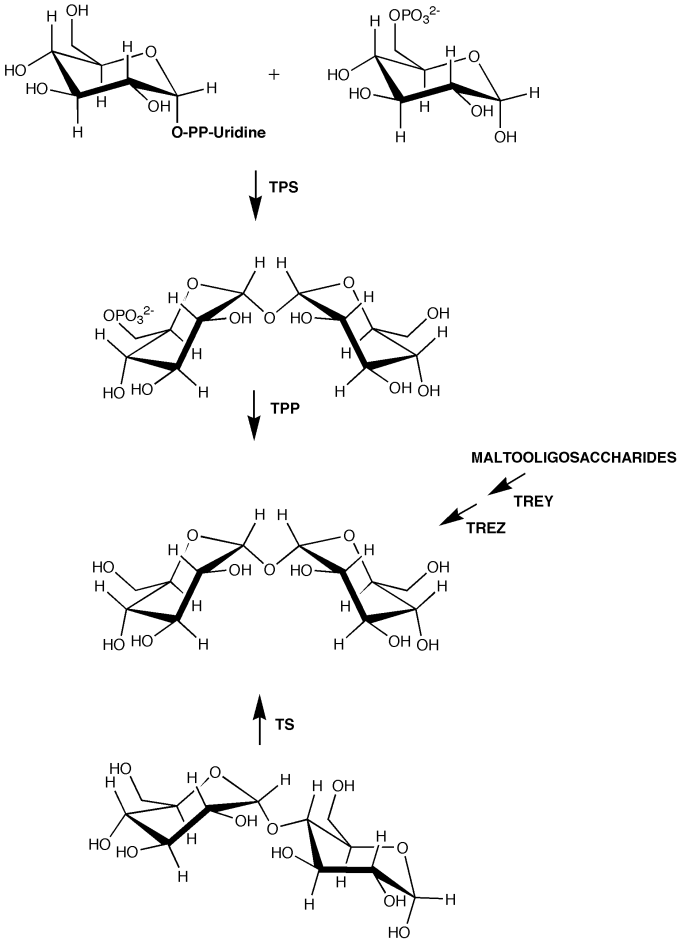

Fig. 2. Pathway of biosynthesis of trehalose in most organisms. This pathway involves the transfer of glucose from UDP-glucose or another glucose sugar nucleotide to glucose-6-phosphate to form trehalose-6-phosphate by the trehalose-P synthase (TPS) and then dephosphorylation of trehalose-P by the specific trehalose-P phosphatase (TPP).

TPS in this review) that catalyzes the transfer of glucose from UDP-glucose to glucose-6-phosphate to produce trehalose-6-P plus UDP (see Figure 2). This reaction was first described in yeast (Cabib and Leloir, 1958) and has since been demonstrated, also from UDP-glucose and glucose-6-P, in numerous organisms, including insects (Candy and Kilby, 1958; Murphy and Wyatt, 1965), *M. tuberculosis* (Lornitzo and Goldman, 1964), and *Dictyostelium discoideum* (Roth and Sussmann, 1966). On the other hand, in *Streptomyces hygroscopicus,* and various other *Streptomyces* species that also produce trehalose-6-P, GDP-glucose, rather than UDP-glucose, serves as the glucosyl donor (Elbein, 1968).

As indicated, the TPSs purified from yeast, insects, or streptomycetes were found to be specific for either UDP-glucose or GDP-glucose as the glucosyl donor, but none of these enzymes could use both donors. However, reexamination of the highly purified TPS from *M. smegmatis* showed that it was active with all of the naturally occurring glucose sugar nucleotides (i.e., ADP-glucose, CDP-glucose, GDP-glucose, TDP-glucose, and UDP-glucose) as glucosyl donors, although UDP-glucose, ADP-glucose, and GDP-glucose were the best substrates (Lapp *et al.*, 1971). For maximum activity with UDP-glucose, the mycobacterial TPS required the presence of a high-molecular-weight polyanion such as heparin, but this polyanion was not necessary when GDP-glucose or ADP-glucose were used as substrates. The 58-kDa protein was purified to apparent homogeneity from *M. smegmatis* and was subjected to amino acid sequencing. Based on that sequence data, the TPS gene was identified in the *M. tuberculosis* genome and was cloned and expressed in *E. coli* as active enzyme.

The recombinant protein showed the same broad substrate specificity with regard to nucleoside diphosphate glucose donors as did the *M. smegmatis* native TPS, demonstrating that this broad substrate utilization was due to a single protein rather than to multiple isozymes with differing specificities. The native and the recombinant TPSs were identical in all other properties, except for the requirement for heparin, which was much lower with the recombinant TPS (Pan *et al.*, 2002). That could be due to the presence of the $(His)_6$-tag on the recombinant enzyme.

The mycobacterial TPS and the plant sucrose synthase may be members of a unique class of glycosyltransferases having rather broad substrate specificities with regard to nucleoside diphosphate glucose donors. Sucrose synthase also can utilize all of the glucose sugar nucleotides for the transfer of glucose to fructose to produce sucrose (Delmer and Albersheim, 1970), with UDP-glucose, ADP-glucose, and TDP-glucose being the preferred substrates. Most other well-studied glycosyltransferases are quite specific for the sugar being transferred, as well as for the purine or pyrimidine base of the sugar nucleotide substrate. This broad substrate specificity of TPS and sucrose synthase may reflect the importance to the organism of these disaccharides (i.e., sucrose and trehalose) as storage compounds, structural components, and stabilizers of cellular structures.

In *S. cerevesiae*, TPS is present as part of a complex that is made up of four subunits. One of these subunits is the synthase (TPS1), another is a specific trehalose-P phosphatase (TPS2, see later discussion), and the other two subunits are thought to be regulatory proteins (TPS3 and TSL1) (Bell *et al.*, 1998). Interestingly enough, *TPS1* is homologous to *otsA* from *E. coli* and also shows about 35% identity over its entire sequence to each of the other yeast subunits in this complex. Why yeast use this enzyme complex to synthesize trehalose, whereas other organisms apparently do not, is not understood, but it may have to do with a regulatory function on trehalose metabolism, and/or on the interaction between trehalose metabolism and glycolysis and fermentation (Noubhani *et al.*, 2000).

In reference to this latter point, workers in this field have proposed the following possible hypotheses to explain this relation between trehalose metabolism and glycolysis:

1. TPS, in addition to synthesizing trehalose-P, may have a regulatory function that restricts glucose influx by its interaction with the glucose transport and sugar kinase activities (Thevelein, 1992);
2. Trehalose metabolism may prevent the overflow of glycolysis by utilizing or diverting sugar-Ps into

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trehalose synthesis, and this pathway then produces inorganic phosphate which is required by glyceraldehyde-3-P dehydrogenase for activity (Hohmann *et al.*, 1993);

3. Trehalose-P may restrict sugar influx into glycolysis by inhibiting hexokinase activity (Blazquez *et al.*, 1993). This study demonstrated that trehalose-6-phosphate competitively inhibited the hexokinases of *Saccharomyces cerevesiae*, with strongest inhibition being against hexokinase II with a K_i of 40 μM.

There is also some evidence for a second pool of TPS in yeast that is present as the free enzyme and is not associated with the complex (Bell *et al.*, 1998). This TPS does not appear to be free as a result of insufficient amounts of the other proteins that make up the complex; in fact, it may have another and distinct role in trehalose metabolism and/or glycolysis. Some recent results indicate that TPS1 mediated protein–protein interactions are involved in control of glucose influx into yeast glycolysis, that trehalose-P inhibition of hexokinase might not be competitive with respect to glucose *in vivo*, and that TPS2 also may play a role in the control of hexokinase activity.

In *E. coli*, two genes governing the synthase were identified and named *otsA* and *otsB*. Mutants unable to synthesize trehalose (*galU*, *otsA*, *otsB*) were osmotically sensitive in glucose-mineral medium (Giaever *et al.*, 1988). The *E. coli otsA* and *otsB* genes were identified and cloned in 1992 (Kaasen *et al.*, 1992). A yeast gene for trehalose-6-P synthase (*otsA*) was cloned in 1993, and it was shown to complement the *E. coli otsA* mutant (Bonini *et al.*, 2000). In addition, the *E. coli otsA* gene was expressed in a *tsp1* yeast mutant that could not grow on glucose. Expression of this gene restored synthesis of trehalose-P and at least a partial ability to grow on glucose (McDougall *et al.*, 1993). Although the genes for TPS and trehalose-phosphate phosphatase (TPP) were characterized in *E. coli*, the proteins themselves have not been well studied and the detailed properties and specificities of these enzymes are not known. There is one report that indicated that OtsA utilized UDP-glucose but not ADP-glucose as the glucosyl donor for trehalose-P synthesis (Giaever *et al.*, 1988). However, other properties of this enzyme have not been detailed nor have the TPS enzymes from plants or insects been well characterized.

Enzymatic conversion of trehalose phosphate to free trehalose

Most cells that contain TPS also have a phosphatase that can convert trehalose-P to free trehalose. Thus, TPP was initially observed in yeast extracts along with the TPS (Cabib and Leloir, 1958), it was partially purified from extracts of *M. smegmatis*, and its specificity for trehalose-P was determined (Matula *et al.*, 1971). The gene for this enzyme is present on the same operon as the TPS gene in *E. coli* (McDougall *et al.*, 1993), and in *S. cerevesiae* TPP is one of the subunits (TPS2) of a multienzyme complex for synthesizing trehalose (Bell *et al.*, 1998). The substrate specificities and other properties of the yeast or *E. coli* TPP have not been well defined.

The *M. smegmatis* gene for TPP was cloned and expressed in *E. coli* as active enzyme with a $(His)_6$ tag at the amino terminus. The expressed enzyme is a 26-kDa protein that shows an almost absolute requirement for Mg^{2+} but is also somewhat active with Mn^{2+}. TPP is specific for trehalose-P as the substrate and has no activity with other phosphate esters, such as glucose-6-P, glucose-1-P, fructose-6-P, glyceraldehyde-3-P, or p-nitrophenyl-phosphate. The *M. smegmatis* enzyme is fairly stable to heating at 60°C and only loses about 10% or 15% of the activity after 5 min at that temperature. This enzyme also maintains its activity in the presence of 0.3% NP-40 or Triton X-100. Circular dichroism studies indicate that the protein has about 50% β-pleated sheet and is very compact (Klutts *et al.*, 2003). Although trehalose-P can be cleaved to free trehalose by nonspecific phosphatases, TPP may have a specific role in regulation or in directing trehalose into a specific functional pathway. Interestingly, two open reading frames (ORFs) in the *M. tuberculosis* genome show about 25% identity at the amino acid level to the TPP of *M. smegmatis* (Klutts *et al.*, 2003). The proteins coded by these ORFs have not yet been identified.

Alternate pathways of trehalose synthesis

Although the TPS/TPP proteins represent the most widely demonstrated pathway for the production of trehalose, several other reactions have been reported in bacteria that produce trehalose. In one case, the enzyme, trehalose synthase (TS), from *Pimelobacter* sp. was shown to catalyze an intramolecular rearrangement of maltose to convert the α1,4-glycosidic linkage of this disaccharide to the α,α1,1-glycosidic linkage of trehalose (Nishimoto *et al.*, 1995). The gene for this TS was cloned from *Pimelobacter* sp. R48 and the sequence analysis revealed a 1719-bp gene, coding a 573-residue amino acid sequence. The 220 N-terminal residues were homologous to those of maltases from *Saccharomyces carlsbergenesis* and *Aedes aegypti* (Nishimoto *et al.*, 1995).

A second alternative pathway for trehalose synthesis in some bacteria is one involving the conversion of maltooligosaccharides or starch to trehalose. Thus thermophilic archaebacteria belonging to the genus *Sulfolobus* apparently have an amylolytic activity that produces glucose and trehalose. The biosynthesis of trehalose was reported to occur in two reactions: maltooligosyltrehalose synthase (TreY) catalyzes the conversion of maltodextrin to maltooligosyltrehalose, and then maltooligosyltrehalose trehalohydrolase (TreZ) hydrolyzes this product to form free trehalose (Maruta *et al.*, 1996b). The reaction catalyzed by TreY is an intramolecular transglucosylation to form the α,α-1,1-glycosidic linkage of trehalose. Three genes in this trehalose biosynthetic pathway were cloned from *Sulfolobus*, that is, *treZ*, *treX*, and *treY*. TreY and TreZ catalyze the reactions described. TreX, a protein of 713 amino acids, codes for a glycogen debranching activity, which is also needed to produce maltodextrins. TreZ is a 556-amino-acid protein, and TreY is a protein of 720 amino acids. These proteins are 33% to 40% homologous to the corresponding enzymes from *Arthrobacter* sp. Q36 (Maruta *et al.*, 1996c).

Because the *M. tuberculosis* genome has been completely sequenced, it is possible to determine whether other sequences have homology to genes within this known genome. This type of analysis was done with the trehalose genes, and

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several ORFs were identified in the *M. tuberculosis* genome that showed considerable homology to the genes encoding enzymes in the three trehalose biosynthetic pathways, already described (DeSmet *et al*., 2000). An ORF in the *M. tuberculosis* genome (Rv0126) had about 71% identity at the amino acid level to the TreS of *Pimelobacter* species. Two other ORFs had considerable homology to other enzymes of the alternate pathways—Rv1563c had about 45% overall identity at the amino acid level with TreY, and Rv1562c had about 53% identity with the TreZ. Some biochemical experiments with cell-free extracts of several mycobacteria provided preliminary evidence for these pathways, but they were not conclusive, nor were attempts to clone and express the genes successful. Because some of the enzymes in the alternative pathways show 50% identity to maltases of yeast and mosquito, it may be that some of the sequence homology in the tuberculosis genome is to maltases rather than to TS or to the TreY.

One other enzyme that could give rise to trehalose is a trehalose phosphorylase that has been reported in mushrooms (Wannet *et al*., 1998). This enzyme catalyzes a reversible reaction that hydrolyzes trehalose in the presence of inorganic phosphate and transfers one glucose to inorganic phosphate to produce glucose-1-phosphate and releases the other glucose as the free sugar (or catalyzes the reverse reaction; i.e., glucose-1-P + glucose to trehalose + Pi). The enzyme, a 240-kDa protein with four identical subunits, has a pH optimum of 6.0 to 7.0. The reaction is reversible, so this enzyme could (theoretically at least), depending on concentrations of reactants and products, be used by these cells to synthesize trehalose from glucose-1-P and free glucose.

Interestingly, *E. coli* can grow on trehalose as the sole source of carbon at either high or low osmolarity. When the cells are placed under conditions of high osmolarity, they synthesize large amounts of trehalose internally as an osmoprotectant. Hoewever, they also can degrade trehalose as the sole source of carbon under both high- and low-osmolarity growth conditions. Thus this bacterium has developed two different systems of trehalose metabolism. Under conditions of high osmolarity, trehalose is hydrolyzed to glucose by a periplasmic trehalase, encoded by *treA*, and the glucose is then taken up by the phosphotransferase system (PTS) as glucose-6-phosphate and used in glycolysis (Boos *et al*., 1990). This periplasmic trehalase, TreA, is induced by 250 mM NaCl but not by trehalose. On the other hand, at low osmolarity, trehalose is transported via a trehalose-specific enzyme II of the PTS system, encoded by *treB*. This trehalose-6-phosphate produced within the cell is then hydrolyzed to glucose and glucose-6-P by trehalose-6-phosphate hydrolase, encoded by *treC* (Rimmele and Boos, 1994).

Hydrolysis or turnover of trehalose

An enzyme hydrolyzing trehalose was first observed in *Aspergillus niger* (Bourquelot, 1893) and then by Fischer in 1895 from *S. cerevesiae* (reviewed in Elbein, 1974). Since that time, trehalase (α,α-trehalose-1-C-glucohydrolase, EC 3.2.1.28) has been reported in many other organisms within the plant and animal kingdoms (Elbein, 1974). Interesting enough, in contrast to other enzymes of trehalose metabolism, trehalase is also found in mammals both in the kidney brush border membranes (Yonemaya and Lever, 1987) and in the intestinal villae membranes (Dahlqvist, 1968). Its role in the kidney is still not clear, but in the intestine its function is undoubtedly to hydrolyze ingested trehalose because individuals with a defect in their intestinal trehalase have diarrhea when they eat foods with a high trehalose content, such as mushrooms (Ruf *et al*., 1990).

The yeast *S. cerevesiae* has been reported to have several different trehalases, one of which is highly regulated, but the exact function of these different enzymes is still not clear. These trehalases are described in more detail later. Trehalose hydrolysis does appear to be an important, perhaps essential process in the life functions of various organisms, such as in fungal spore germination, insect flight, and the resumption of growth in resting cells (Nwaka and Holzer, 1998).

In 1982, an inactive form of trehalase (zymogen) was reported in *S. cerevesiae* that became activated by a cyclic AMP-dependent phosphorylation (Thevelein, 1984). This cAMP-activated enzyme was found to be located in the cytoplasm. However, a second trehalase activity was identified in the vacuoles of these cells, and this latter enzyme was constitutively active (Londesborough and Varimo, 1984). The two different trehalases were separated and partially characterized: the cytoplasmic enzyme had a pH optimum of about 7 and was called the neutral trehalase, whereas the vacuolar protein exhibited maximal activity at a pH of 4.5 and was referred to as the acid trehalase (van Solingen and van der Plaat, 1975; Keller *et al*, 1982).

The neutral cytosolic trehalase was purified to homogeneity and exhibited a mass of 160 kDa on nondenaturating gels and a mass of 80 kDa by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). This hydrolase was specific for trehalose as the substrate, and was inactive on cellobiose, maltose, lactose, sucrose, raffinose, and melibiose (App and Holzer, 1989). The K_m of the enzyme for trehalose was about 35 mM, but because trehalose may be present in yeast at up to 23% of the dry weight, this enzyme may have considerable physiological significance in maintaining or decreasing trehalose concentrations (Dellamora-Ortiz *et al*., 1986). This may be especially important in yeast, in which trehalose has been shown to act as a regulator of metabolism and may affect the activity of key enzymes such as hexokinase. The activity of the neutral trehalase in crude extracts is enhanced by polycations, but the activity of the purified phosphorylated enzyme is inhibited by polycations. The activation in crude extracts was found to be due to removal of RNA and polyphosphates, both of which inhibit the neutral trehalase. This suggested to these workers that a second type of activation mechanism might exist for this enzyme that involves the removal of an inhibitor by the polycations (Nwaka and Holzer, 1998). Dephosphorylation of the purified, phosphorylated neutral trehalase by alkaline phosphatase caused an almost complete inactivation of the enzyme, whereas rephosphorylation by incubation with protein kinase and ATP resulted in recovery of the activity. The amino acid that became phosphorylated in this reaction was serine (App and Holzer, 1989).

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The acid or vacuolar trehalase was also purified and showed a rather diffuse band on SDS gels with a molecular weight range of 167–265 kDa and a molecular weight of 218 kDa by high-performance gel filtration (Londesborough and Varimo, 1984). The broad molecular weight smear observed by SDS–PAGE is due to the high carbohydrate content of this protein as shown by treatment with endo-β-N-acetylglucosaminidases (Mittenbuhler and Holzer, 1988). The acid enzyme had a pH optimum of about 4.5 and was also very specific for trehalose as the substrate. Thus the enzyme showed no activity with maltose, cellobiose, mellibiose, sucrose, or lactose (Mittenbuhler and Holzer, 1988). This trehalase had a K_m for trehalose of about 4 mM, which is considerably lower than that of the neutral trehalase. Deletion of the gene for this protein leads to an inability of yeast cells to grow on trehalose as a carbon source (Nwaka *et al.*, 1996), indicating a key role for this trehalase in trehalose utilization. One suggestion is that this trehalase moves from its site of synthesis in the endoplasmic reticulum/Golgi to the periplasmic space, where it binds exogenous trehalose to internalize it and cleave it in the vacuoles to produce free glucose (Stambuk *et al.*, 1996). It has been reported that the activities of the neutral trehalase and the acid trehalase are low in yeast cells growing exponentially but high during stationary phase growth after glucose has been depleted (Winkler *et al.*, 1991). In addition, the expression of the genes for these enzymes appear to be low in exponentially growing cells as compared with stationary phase cells, suggesting that these genes are repressed by glucose (Nwaka *et al.*, 1995).

Expression of the neutral trehalase gene is low in exponentially growing yeast cells as compared with stationary phase cells, suggesting that the gene is repressed by glucose. Such a dependence of gene expression on the presence of glucose in the culture medium is typical of genes that are under the control of catabolite repression and suggests a role for this enzyme in glucose metabolism (Nwaka and Holzer, 1998). In keeping with this catabolite repression of trehalase expression, stationary phase cells of yeast have increased levels of cytoplasmic trehalose and also increased levels of trehalase activity. This high expression of trehalases in stationary phase as well as high levels of trehalose may contribute to the high stress tolerance of these cells.

As indicated earlier, various bacteria, such as *E. coli*, have trehalases that may function as part of an uptake system such as to supply glucose to the PTS (Horlacher *et al.*, 1996). They also have trehalase activity that may be involved in the function of trehalose as an osmoregulator.

Trehalase is obviously present in many different organisms, but in most cases neither the function nor the properties of this important enzyme have been studied. In some plants, such as *Arabidopsis*, trehalase has been proposed to protect the plants from the growth inhibition induced by exogeneously supplied trehalose, such as that presented to plants by soilborne microorganisms. That is, these enzymes may also be important to degrade exogenously produced trehalose (Muller *et al.*, 1999). Because high concentrations of trehalose may cause problems with osmolarity, inhibit key metabolic reactions, or be toxic in other ways, trehalase may be of considerable importance in regulating the levels of trehalose in the cell. As will be discussed, the levels of trehalose are greatly increased in various cells when they are exposed to a variety of environmental stresses; therefore, in some cases trehalase may play a role in removing trehalose and bringing the cell back to homeostasis once the stress is over. Certainly this important enzyme deserves more investigation to understand its role in energy and in carbon metabolism and in understanding the various roles of its major substrate, trehalose.

Functions of trehalose

As an energy and carbon reserve

As discussed earlier, trehalose levels may vary greatly in certain cells depending on the stage of growth, the nutritional state of the organism or cell, and the environmental conditions prevailing at the time of measurement. In insects, trehalose is a major sugar in the hemolymph and thorax muscles and is consumed during flight (Becker *et al.*, 1996). Trehalose is also an important component in fungal spores, where trehalose hydrolysis is a major event during early germination and presumably serves as a source of carbon for synthesis and glucose for energy (Thevelein, 1984; Rosseau *et al.*, 1972).

As a stabilizer and protectant of proteins and membranes

Protection from dehydration. Although water is obviously necessary for life, some organisms are able to survive almost complete dehydration, for instance, even when 99% of their water content is removed. This includes common organisms such as plants, yeast cells, and fungal spores, but also microscopic animals such as nematodes, rotifers, and the cysts of brine shrimp (Leopold, 1986). Some of these dried organisms may remain in this state, known as anhydrobiosis, for decades under favorable conditions until water becomes available. When that happens, these organisms swell and resume the active state. Recent studies on these organisms have demonstrated mechanisms that allow them to survive dehydration; understanding some of these mechanisms have enabled researchers to develop new methods for the preservation of biological materials that are normally sensitive to drying (Crowe *et al.*, 1992).

Anhydrobiotic organisms generally contain high concentrations of trehalose (and sometimes other disaccharides and oligosaccharides). Thus it was shown that when the nematode *Aphelenchus avenae* was slowly dehydrated, it converted as much as 20% of its dry weight into trehalose (Madin and Crowe, 1975). The ability of this organism and others to survive in the absence of water has shown a strong correlation with the synthesis of trehalose (Madin and Crowe, 1975; Womersley, 1981). Log-phase cultures of yeast have low concentrations of trehalose and are quite susceptible to dehydration, but as they enter the stationary phase of growth the levels of trehalose increase, along with their ability to survive dehydration (Gadd *et al.*, 1987). This ability to survive in the presence of trehalose is independent of the growth phase of the cells because log-phase cells subjected to heat shock rapidly synthesize trehalose and also acquire the ability to survive dehydration (Hottinger *et al.*, 1987). These results are also applicable to a variety of

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other organisms ranging from brine shrimp (Leopold, 1986) to the resurrection plant (Zentella *et al*., 1999). This use of trehalose to enable cells to survive dehydration (and other stresses) may be an ancient adaptation because even *Archaebacteria* have been found to accumulate trehalose in response to stress (Nicolaus *et al*., 1988). Interestingly, in plants the disaccharide sucrose plays a similar role to that of trehalose in yeast and nematodes (Anandarajah *et al*., 1990). According to Leopold (1986), trehalose is preferred over sucrose by most organisms because it has less tendency to form crystals than does sucrose.

The two primary stresses that are proposed to destabilize lipid bilayers during dehydration are fusion and lipid phase transitions. Studies by laser light scattering or other techniques demonstrate that trehalose and other sugars inhibit fusion between the vesicles during drying, but the inhibition of fusion alone is not sufficient to preserve the dry vesicles. Thus trehalose is also necessary to prevent phase transitions (Crowe and Crowe, 1990). The evidence suggests that trehalose depresses the phase transition temperature of the dry lipids, which maintains them in the liquid crystalline phase in the absence of water (Crowe and Crowe, 1988). A large body of evidence indicates that the stabilizing effect of trehalose is due to its structure and stereochemistry. X-ray diffraction studies show that trehalose fits well between the polar head groups with multiple sites of interaction and suggests that the strong stabilizing effects of trehalose are related to its stereochemistry, which provides the most favorable fit with the polar head groups (Rudolph *et al*., 1990).

Trehalose also preserves labile proteins during drying. For example, phosphofructokinase is a tetramer that irreversibly dissociates to inactive dimers during the drying process. Although many compounds that stabilize proteins can prevent this dissociation when excess water is still present, only disaccharides are effective in stabilizing this protein during extreme drying. As in the case of membranes, trehalose probably interacts directly with the dry protein by hydrogen bonding between its hydroxyl groups and polar residues in the protein (Carpenter and Crowe, 1989). It is not clear why this interaction results in stabilization, but the fact that trehalose is a nonreducing sugar means that it cannot undergo the typical browning reaction between the aldehyde group on reducing sugars and amino groups on proteins. The browning reaction usually leads to denaturation of proteins.

An increased resistance to withstand drought conditions has been conferred on several different plants and also animal cells by increasing their levels of intracellular trehalose. For example, transgenic tobacco plants were engineered to produce substantial amounts of trehalose by introducing the *E. coli otsA* and *otsB* genes for trehalose synthesis. The introduction of these genes had a pronounced effect on plant morphology and growth performance under drought conditions. Thus the transgenic plants showed considerably improved growth (Pilon-Smits *et al*., 1998). The same 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 controls cells that were very sensitive to drying (Guo *et al*., 2000).

Protection against heat. In yeast, stimuli that trigger the heat shock response also cause the accumulation of trehalose. In fact at least two subunits of the trehalose-6-P synthase complex of *S. cerevesiae* are actively synthesized during heat shock (Bell *et al*., 1992), and physiological concentrations of trehalose (up to 0.5 M) were found to protect enzymes of yeast and other organisms from heat inactivation *in vitro*. Trehalose also reduced the heat-induced formation of protein aggregates. Trehalose was as good or better as a protein stabilizer than any of a number of compatable solutes, including polyols, sugars, and amino acids (DeVirgilio *et al*., 1994). Yeast mutants were prepared that were defective in genes coding for key enzymes in trehalose metabolism (*TPS1*, *TPS2*), and these mutants showed an inability to accumulate trehalose on mild heat shock and were much less resistant to heating than was the wild-type organism. These various studies strongly implicate trehalose as playing a key role in thermotolerance and also indicate that the enzymes that synthesize trehalose are induced in response to the stress in order to increase the levels of trehalose.

An important *in vivo* and *in vitro* study showed that trehalose protects cells from heat by stabilizing proteins at high temperatures. Using two different temperature-sensitive reporter proteins, these investigators showed that enzymes are better able to retain activity during heat shock in cells that are producing trehalose (Singer and Lindquist, 1998). These studies showed an additional and important role of trehalose, that is, the ability to suppress aggregation of proteins that have already been denatured. Based on these studies, these researchers also explained why trehalose must be degraded rapidly after the heat shock has ended, that is, if the unfolded luciferase, one of their reporter proteins, is removed from or diluted out of the trehalose, it can be refolded by molecular chaperones. On the other hand, if the trehalose concentration remains high, it interferes with the refolding process, and the protein is not renatured by the chaperone (Singer and Lindquist, 1998). Thus, it may be important to have active trehalase present once the heat stress is alleviated.

Protection from damage by oxygen radicals. Another role for trehalose is in protecting cells against oxygen radicals. Exposure of *S. cerevesiae* to a mild heat shock or to a proteosome inhibitor induced trehalose accumulation (as indicated) and also markedly increased the viability of cells on exposure to a free radical–generating system (H_2O_2/iron). However, when the cells were returned to the normal growth temperature, both the trehalose content and the resistance to oxygen stress decreased rapidly and returned to the wild-type level. A mutant cell line defective in trehalose synthesis was much more sensitive to oxygen killing than was the wild type, but adding trehalose to the medium enhanced the resistance of these cells to H_2O_2. The effect of oxygen radicals on these cells was to damage amino acids in cellular proteins and the presence of high concentrations of trehalose in the cells prevented this damage. The trehalose-defective mutant showed a much higher content of damaged proteins even after only a brief exposure to oxygen stress. The suggestion is that trehalose acts as a free radical scavenger. In these studies, mannitol and galactose also protected but less so than trehalose, whereas

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sucrose was ineffective. This lack of protection by sucrose may have to do with its inability to quench oxygen radicals or be taken up by cells (Banaroudj *et al.*, 2001).

As indicated earlier in this review, trehalose and sucrose are both nonreducing disaccharides that may have considerable similarity in synthesis and function. Both are stored in the cytosol of cells, and both may be present in their respective cells in high concentrations depending on various environmental conditions. Another commonality is that when either of these oligosaccharides are present in high concentration, the cells become quite resistant to a variety of stress conditions, including heat, dehydration, oxygen stress, and so on (Hincha *et al.*, 2002). In plants, oligosaccharides of the raffinose series (Galα1-6Sucrose and higher) can accumulate in cells to a level of 15% of the dry weight, and these plants may have considerable stress resistance (Hincha *et al.*, 2002). This striking correlation cannot be ignored and needs further investigation to determine whether raffinose and stachyose and other sucrose oligosaccharides are in fact produced as a protection against different stress conditions. The same may be true of the trehalose oligosaccharides that have been isolated from the cytosol of *M. smegmatis* (Ohta *et al.*, 2002). Although their concentrations in these cells were fairly low, that could be because those cells were not stressed. It will be important to examine the levels of these various trehalose analogs after cells have been exposed to stress.

Protection from cold. A mutant strain of *E. coli* that was unable to produce trehalose died much faster that did the wild type at 4°C. However, transformation of this mutant with *otsA*/*otsB* genes restored the ability to synthesize trehalose and also cell viability in the cold (Kandror *et al.*, 2002). Additional studies showed that downshifting cells from 37°C to 16°C caused an eightfold increase in trehalose levels and a marked increase in mRNA levels for otsA and otsB. The authors speculate that because a number of proteins denature and precipitate in the cold where the hydrophobic effects are relatively weak, it is possible that trehalose also prevents the denaturation and aggregation of specific proteins at cold temperatures. Trehalose may also stabilize cell membranes whose fluidity decreases during temperature downshift. Thus, exogenous trehalose has been shown to protect a variety of organisms against freezing, with maximum protection seen when trehalose is present on both sides of the membrane.

As a sensing compound and/or growth regulator

Although tobacco plants transformed with the genes for enzymes of trehalose synthesis (TPS or TPP) do exhibit a slight increase in drought tolerance (still somewhat controversial), the expression of the microbiol genes for trehalose synthesis causes severe growth defects, such as dwarfism and aberrant root development (Vogel *et al.*, 1998). These findings have led these researchers to postulate that trehalose or related metabolites might function as regulators of plant growth and development. This effect could be similar to the effect of trehalose-6-P on hexokinase and glycolysis in yeast, or it could be due to an effect on other metabolic pathways. Interestingly, similar growth defects were observed with transformed rice plants even though these plants did not accumulate large amounts of trehalose (Muller *et al.*, 1999). The authors provide three possible explanations as follows: (1) the pleiotropic phenotype may be due to a disturbance of normal plant metabolism, such as exhaustion of UDP-glucose; (2) even the small amounts of trehalose or trehalose-P might be toxic to plants; or (3) trehalose metabolism may be a signal in sugar sensing and partitioning of assimilates.

As a structural component of the bacterial cell wall

In mycobacteria and corynebacteria, trehalose is the basic component of a number of cell wall glycolipids (Lederer, 1976). The best known and most widely studied of these trehalose lipids is cord factor, a cell wall lipid of *M. tuberculosis* that contains the unusual fatty acid mycolic acid esterified to the 6-hydroxyl group of each glucose to give trehalose-dimycolate. This lipid is considered to be one of the major toxic components of the cell wall and is also largely responsible for the low permeability of the mycobacterial cell wall, which confers considerable drug resistance to these organisms (Brennan and Nikaido, 1995). Trehalose-monomycolate is the proposed precursor to trehalose-dimycolate (cord factor), but it also appears to serve as the donor of mycolic acid residues to the cell wall arabinogalactan to form the mycolyl-arabinogalactan-peptidoglycan complex (Chatterjee, 1997). A mycolyl transferase was isolated, and this enzyme was shown to catalyze the transfer and exchange of mycolic acid from trehalose-monomycolate to free trehalose to produce both mono- and dimycolyl-trehalose (Belisle *et al.*, 1997). Whether this enzyme or a similar transferase is involved in transferring mycolic acid residues to cell wall polymers remains to be determined. Corynebacteria and nocardia also contain trehalose glycolipids that have fatty acids that are related to but not identical with the mycolic acids, and these fatty acids are referred to as corynomycolic or nocardomycolic acids (Lederer, 1976). The function of these lipids, besides their obvious structural role, is not known.

There are other antigenic glycolipids in the mycobacterial cell wall that also have trehalose as the base. For example there are a variety of acylated-trehaloses that have three major types of fatty acids attached to the 2 and 3 hydroxyl groups of the same glucose. These fatty acids are: n-C_{16-19} saturated fatty acids, C_{21-25} α-methyl branched fatty acids, and C_{24-28} α-methyl branched, β-hydroxy fatty acids (Besra *et al.*, 1992). *M. tuberculosis* and other mycobacteria also have trehalose lipids that contain sulfate, such as 2,3,6,6′-tetra-acyl-2-sulfate trehalose (sulfatide I) (Alugupalli *et al.*, 1995), or other types of fatty acids, such as phthienoic acids (Daffe *et al.*, 1988). This great variation in the types of fatty acids found in these organisms and as cell wall components suggests a probable function, but so far specific functions have not been demonstrated.

Finally, mycobacteria, such as *Mycobacterium kansasii*, is characterized by the presence of seven species-specific neutral lipooligosaccharide antigens. These oligosaccharide antigens have a common tetraglucose core which is distinguished by an α,α-trehalose substituent to which are linked such various sugars as xylose, 3-O-methylrhamnose, fucose, and a novel N-acyl aminosugar. The exact structures of these compounds has not been determined. Analogous but

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specific lipooligosaccharides typify a host of other atypical mycobacteria (Hunter *et al*., 1983).

Conclusion

Trehalose is a nonreducing disaccharide of glucose that is widespread throughout the biological world, being found in bacteria, fungi, yeast, insects, nematodes, shrimp, plants, and probably many other organisms. It does not occur in mammalian cells, although humans have the enzyme trehalase in intestinal villae cells and in kidney brush border cells, probably to handle ingested trehalose. Many of these lower organisms produce and store trehalose, sometimes in amounts as high as 10% to 20% of their dry weight. The formation of trehalose is induced by stress conditions such as heat, drying, oxidative stress, and so on, and convincing evidence indicates that this accumulated cytoplasmic trehalose protects proteins and membranes from denaturation caused by these stresses. In addition, trehalose may act as a signaling or regulatory molecule in some cells and link trehalose metabolism to glucose transport and glycolysis. Finally in some bacteria, especially actinomycetes, trehalose is a basic component of a number of different glycolipids that are cell wall components. One of these glycolipids, trehalose-dimycolate, or cord factor, is one of the toxic components of the tubercle bacillus cell wall. Its precursor, trehalose-monomycolate, appears to function as a donor of mycolic acid to form mycolyl-arabinogalactan. Although these are proposed roles for trehalose and some have substantial evidence to support them, the mechanisms of how trehalose acts as a signaling molecule or how it protects proteins and membranes from damage remain to be established. Furthermore, the physiological function of the various types of trehalose glycolipids that are found in the cell walls of mycobacteria, corynebacteria, and nocardia need to be determined. Finally, one must acknowledge that there may be other functions for this simple but very diverse sugar that have not yet been determined. Additional studies on trehalose are needed to address these unknowns.

Abbreviations

ORF, open reading frame; PTS, phosphotransferase system; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TPP, trehalose-phosphate phosphatase; TPS, trehalose-phosphate synthase; TreX, glycogen debranching enzyme; TreY, maltooligosyltrehalose synthase; TreZ, maltooligosyltrehalose trehalohydrolase; TS, trehalose synthase.

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