Biochemical and genomic regulation of the trehalose cycle in yeast: review of observations and canonical model analysis

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Abstract

The physiological hallmark of heat-shock response in yeast is a rapid, enormous increase in the concentration of trehalose. Normally found in growing yeast cells and other organisms only as traces, trehalose becomes a crucial protector of proteins and membranes against a variety of stresses, including heat, cold, starvation, desiccation, osmotic or oxidative stress, and exposure to toxicants. Trehalose is produced from glucose 6-phosphate and uridine diphosphate glucose in a two-step process, and recycled to glucose by trehalases. Even though the trehalose cycle consists of only a few metabolites and enzymatic steps, its regulatory structure and operation are surprisingly complex. The article begins with a review of experimental observations on the regulation of the trehalose cycle in yeast and proposes a canonical model for its analysis. The first part of this analysis demonstrates the benefits of the various regulatory features by means of controlled comparisons with models of otherwise equivalent pathways lacking these features. The second part elucidates the significance of the expression pattern of the trehalose cycle genes in response to heat shock. Interestingly, the genes contributing to trehalose formation are up-regulated to very different degrees, and even the trehalose degrading trehalases show drastically increased activity during heat-shock response. Again using the method of controlled comparisons, the model provides rationale for the observed pattern of gene expression and reveals benefits of the counterintuitive trehalase up-regulation.

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1. Introduction

Trehalase is a non-reducing disaccharide that has been found in bacteria, eukaryotic microorganisms, plants, insects and invertebrates, but so far not in mammals (Benaroudj et al., 2001). It has been studied extensively in the baker’s yeast, \textit{Saccharomyces cerevisiae}, where it was originally thought to serve as a carbohydrate reservoir like glycogen (Panek and Mattoon, 1977; Lillie and Pringle, 1980). Trehalose is now being recognized as a crucial defense mechanism that stabilizes proteins and biological membranes under a variety of stress conditions, including increased temperature, hydrostatic pressure, desiccation, nutrient starvation, osmotic or oxidative stress, and exposure to toxic chemicals (Crowe et al., 1984; Attfield, 1987; van Laere, 1989; Wiemken, 1990; De Virgilio et al., 1994; Hottiger et al., 1994; Reinders et al., 1997; Hounsa et al., 1998; Singer and Lindquist, 1998a; Iwahashi et al., 2000; Benaroudj et al., 2001). The trehalose system is also important for the control of glucose influx during the cellular response to adverse conditions (Thevelein and Hohmann, 1995; Bonini et al., 2000), and the gene of one of its production enzymes (TPS1) shows strong homology with GGS1, a gene that is associated with a glucose-sensing complex and with transport of glucose into the cell (Thevelein and Hohmann, 1995). Based on its unique properties of stabilizing molecules, its mild sweetness, high solubility, low hygroscopicity and, last but not least, a price that has become affordable through genetic modifications of microorganisms, trehalose has become an important target...
for biotechnology, where it is produced for food manufacturing, vaccine protection in hot climates, and cosmetic products, such as lip sticks (Schiraldi et al., 2002).

The trehalose pathway consists of only a few metabolites, which form a substrate cycle, and is governed by a surprisingly complex control system that comprises several inhibiting or activating signaling mechanisms. The design and operation of the tightly controlled trehalose cycle are difficult to explain with casual reasoning and cannot even readily be addressed with an experimental approach, because targeted changes in the susceptibility of a given enzymatic step to induction, repression, inhibition or activation are not always easy to implement without causing other changes or decreased viability. As an effective alternative, it is shown here how mathematical modeling can shed light on the regulation, design, and operation of a biochemical pathway, as exemplified with the trehalose cycle. To this end, the trehalose cycle is translated into a canonical model according to the tenets of Biochemical Systems Theory (BST; Savageau, 1969a, b).

The rational construction of the canonical model requires first a review of key observations on the regulatory features of the trehalose cycle in wild type and mutants. Once the model is symbolically and numerically defined, a first baseline analysis assesses whether the model is reasonable, as judged by its steady-state and dynamical properties. Second, model responses computed for simulated environmental conditions of abundant or low-glucose conditions reveal the degree of model consistency with experimental observations. Third, the model allows evaluation of the specific roles of regulatory signals for the proper functioning of the pathway. The primary tool for this part of the analysis is the method of controlled mathematical comparisons (MCMC), which is an integral part of BST (Savageau, 1985; Irvine, 1991; Alves and Savageau, 2000) and explained in a later section.

Following the analysis of the model under normal conditions, the paper addresses the operational details of the cell’s response to heat shock. For this purpose, all enzyme activities are altered in accordance with observed changes in gene expression after heat shock (Stanford Database, 2003). The model analysis of this situation is executed in three steps. First, the heat-shock model is again tested with respect to steady-state and dynamical features. Second, the consequences of each alteration in activity are investigated by comparing the heat-shock model with an alternative model without that particular alteration. Third, this controlled comparison allows us to associate specific changes in enzyme activity with consequent physiological responses.

It seems that the model presented here is the first systematic attempt to integrate genomic, biochemical, and physiological information of the trehalose system into one functioning entity. The results and discussion of this integrative analysis illustrate that, even for a small pathway, the simultaneous, quantitative consideration of experimental observations from all three levels of organization is necessary for gaining a clear picture of its intricate inner workings.

2. Biological background

Trehalose (α-D-glucopyranosyl α-D-glucopyranoside or α,α-1,1-diglucose) is produced in a multi-step process, whose substrate is glucose (Fig. 1). Glucose is converted into glucose 6-phosphate (G6P) which, together with uridine diphosphate glucose (UDPG), leads to the formation of trehalose 6-phosphate (T6P) and subsequently trehalose. Trehalose can be split into two

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**Fig. 1.** Diagram of trehalose cycle in *S. cerevisiae*. Solid arrows show flow of material, with thickness indicating the magnitude of flux. Dotted arrows represent inhibitory (−) and activating (+) signals.
molecules of glucose, thus closing the trehalose cycle: 2 glucose → G6P + UDPG → T6P → trehalose → 2 glucose.

Glucose is taken up from the medium and immediately phosphorylated and further metabolized, with the consequence that glucose transport into the cell is the most influential step of trehalose production (Aon et al., 1995). In yeast, 20 transporters are involved in glucose uptake. Their quantities and activities are determined by the external glucose conditions through transcriptional regulation of the various transporter genes; Özcan and Johnston (1999) recently provided a comprehensive review. It is commonly assumed that glucose transport into the cell is inhibited by G6P (e.g. Galazzo and Bailey, 1990).

Three enzymes (hexokinase I, hexokinase II, and glucokinase) catalyse the irreversible hexokinase step; their corresponding genes are HXK1, HXK2, and GLK1 (e.g. Walsh et al., 1991; Rodríguez et al., 2001). The three enzymes control each other through complex interactions that determine which kinase is most active under given glucose conditions. For instance, if yeast is grown on non-fermentable carbon sources, such as ethanol or galactose, the mRNA abundance for HXK2 is extremely low, but when glucose is available, hexokinase I mediates the expression of HXK2. Some of the generated protein (hxk2) moves into the nucleus, where it is involved in the repression HXK1 and GLK1 and in further expression of HXK2 (Rodríguez et al., 2001). Thus, during exponential growth on glucose, hexokinase I and glucokinase are down-regulated, whereas hexokinase II is induced and becomes the dominant isoenzyme with about 15,000 molecules per cell (Gancedo et al., 1977). This induction is readily reversed: after a switch from glucose to ethanol, the mRNA level for hexokinase II decreases from abundant to undetectable within 30 min (Herrero et al., 1995). The enzymes themselves are very stable in vivo and, without further transcription, remain active for several hours (Panneman et al., 1998). Relevant to the present context is that T6P is a strong inhibitor of hexokinase II at physiological concentrations, but does not inhibit glucokinase at all, and hexokinase I only weakly (Blázquez et al., 1993; Panneman et al., 1998; Boy-Marcotte et al., 1999).

G6P is the key metabolite of sugar metabolism (DeRisi et al., 1997). Under normal conditions, roughly two-thirds of it is used for glycolysis (Galazzo and Bailey, 1990), about 10% is channeled toward the pentose phosphate pathway, about 20% is used for the readily reversible conversion to G1P, and small quantities are used for the production of T6P and other pathways. Most interesting is the activation of the various branches at G6P in response to heat shock (Stanford Database, 2003). Phosphofructokinase, the first committed step of the glycolytic branch is not up-regulated at all, whereas the pathways toward trehalose and glycogen are strongly up-regulated (10–20-fold), and the pentose branch is up-regulated about six-fold. This differential pattern is an indication of the crucial channeling of sugars at the G6P branch point and will be discussed in a later section.

The formation of trehalose occurs in a two-step process from G6P and UDPG (Cabib and Leloir, 1958; Vandercammen et al., 1989). First, a hexosyl group transfer leads to the formation of T6P, which through phosphoric ester hydrolysis is subsequently converted to trehalose by a specific T6P phosphatase. The two involved enzymes form a complex (Bell et al., 1998), which is composed of subunits encoded by the genes TPS1, TPS2, TPS3 and TSL1 (“trehalose phosphate synthase” and “trehalose synthase long chain”) that are co-regulated and induced by heat (Winderickx et al., 1996). The T6P synthase reaction is irreversible, at least in bacteria (Lapp et al., 1971). Its gene, TPS1, is repressed by glucose (Lutfiyya et al., 1998), and the level of glucose repression determines the concentration and state of activation of the trehalose production complex (Panek and Mattoon, 1977; Hottiger et al., 1987). By contrast, the phosphorylated substrates of the process, G6P and UDPG, induce trehalose production (Lapp et al., 1971; Vandercammen et al., 1989). TPS1 induction after heat shock is accompanied by corresponding changes in the amount of product, T6P (Vuorio et al., 1993; Parrou et al., 1997, 1999). Whereas the corresponding enzymes T6P synthase (tps1) and T6P phosphatase (tps2) carry the catalytic activity of trehalose synthesis, tps3 and tsl1 seem to be important for the integrity of the trehalose synthase complex and to play a purely regulatory role without obvious enzymatic activity (Reinders et al., 1997; Thevelein and Hohmann, 1995; Bell et al., 1992; Vuorio et al., 1993; Parrou et al., 1997).

Neutral and acid trehalases convert trehalose into two molecules of glucose through O-glycosyl bond hydrolysis (Brenda, 2003). They are located in different cellular compartments and exhibit different activity profiles during growth (San Miguel and Argüelles, 1994; Zähringer et al., 1997). To what degree they are otherwise regulated in the same manner is not clear, but expression experiments have shown that both are strongly and simultaneously up-regulated after moderate heat shock (Stanford Database, 2003). As a null-mutation in the neutral trehalase gene NTH1 demonstrates, this step is crucially important for trehalose mobilization (e.g. De Virgilio et al., 1994), because deficiency in trehalase activity leads to an accumulation of trehalose, which impedes recovery from heat shock (e.g. De Virgilio et al., 1991b). Conversely, overproduction of trehalase results in insufficient trehalose concentrations under stress and correspondingly insufficient protection of proteins, and this may increase mortality (Espinet et al., 1995). Glucose
represses trehalase via the cAMP–PKA pathway (Thevelein and Hohmann, 1995; Thevelein and de Winde, 1999), but apparently only during the transition from gluconeogenesis and respiratory growth to fermentative growth (Vanhalewyn et al., 1999). This situation is not considered in the present context, and the regulation is neither shown in Fig. 1 nor considered in the model. The role of the cAMP–PKA pathway is, however, described in greater detail in a later section.

Under normal physiological growth conditions and a temperature of about 25°C, T6P and trehalose are found only in trace concentrations. Glucose is used primarily for glycolysis, and any excesses are stored in the form of glycogen, which, like trehalose, is produced from UDPG (see Fig. 1), but constitutes a carbohydrate storage that is energetically superior to trehalose (Wiemken, 1990). If the temperature rises above 37°C, almost all genes associated with the trehalose cycle are up-regulated, but to noticeably different degrees (Fig. 2), spanning a range from essentially unchanged to 150-fold (Stanford Database, 2003). Subsequently, the abundance of mRNA increases rapidly and strongly (De Virgilio et al., 1991; Nwaka et al., 1995a; Parrou et al., 1997), the activity of T6P synthase and T6P phosphatase increases several fold (De Virgilio et al., 1991a; Neves and François, 1992), and the amount of trehalose may rise to an amazing 500 mM or 1 g/g protein (Wiemken, 1990; Hottiger et al., 1987, 1994; Ertugay and Hamamci, 1997), with the final concentration depending on temperature. This dramatic response is apparently vital, because mutants lacking the ability to form trehalose exhibit increased sensitivity to environmental stresses, including heat (e.g. De Virgilio et al., 1991a, 1994; Argüelles, 1994; Hounsa et al., 1998; Singer and Lindquist, 1998b; Benaroudj et al., 2001). Similarly, overproduction of trehalase can be lethal, because it leads to increased trehalose degradation and thus to a reduced trehalose concentration (Espinet et al., 1995).

Trehalose plays several important roles in the response to heat shock. Foremost, it protects proteins against heat-induced destabilization and denaturation and reduces the formation of protein aggregates (Winkler et al., 1991; Argüelles, 1994; Singer and Lindquist, 1998b). Similarly, trehalose apparently protects DNA and lipids (Benaroudj et al., 2001) and preserves the integrity of hydrated membranes by substituting water molecules and binding to the polar head groups of phospholipids (Crowe et al., 1984; Argüelles, 1994). In addition to protecting macromolecules and membranes, the trehalose cycle is involved in the control of glucose. Without sufficient amounts of trehalose, heat shock leads to an accumulation of free cytoplasmic glucose and sugar phosphates, which results in an overloading of glycolysis and depletion of cytoplasmic phosphate and ATP (Hohmann et al., 1993; Neves et al., 1995; Ernandes et al., 1998; van Vaeck et al., 2001). While insufficient quantities of trehalose are detrimental under heat-shock conditions, an excess of trehalose is just as problematic. Not only does hyperaccumulation of trehalose not improve survival (Hounsa et al., 1998), the rapid mobilization and removal of trehalose is absolutely critical for successful recovery from heat shock, because high concentrations of trehalose impede the refolding of proteins that had partially denatured during heat stress (Singer and Lindquist, 1998a, b; Wera et al., 1999). Two review articles (Nwaka and Holzer, 1998; François and Parrou, 2001) provide details.

Boy-Marcotte et al. (1999) hypothesized that an important function of the trehalose cycle could be to increase the intracellular level of T6P, which is a potent inhibitor of hexokinase II. They argued that the subsequent replacement of hexokinase II with

![Diagram](image-url)
hexokinase I and glucokinase could play a crucial role in the control of the glycolytic flux and neighboring pathways, such as the pentose phosphate pathway. However, while the activity of T6P synthase is increased several times under heat shock (Hottiger et al., 1987; Stanford Database, 2003), there is not much change in T6P concentration (Blázquez et al., 1993; Winkler et al., 1991). A probable explanation is that the genes responsible for T6P production and degradation are induced in a similar fashion and to the same extent (Parrou et al., 1997), and that the two enzymes are part of the same complex, so that the influx–efflux balance through the T6P pool remains more or less constant. Also, T6P is toxic at high concentrations (Sur et al., 1991; Thevelein and Hohmann, 1995).

Although not part of the trehalose cycle, it is imperative to consider the formation and degradation of glycogen, because it serves as a carbohydrate storage that is formed from UDPG (e.g. Wiemken, 1990; Parrou et al., 1997) and is thus in direct competition with the production of trehalose. Glycogen in yeast is disassembled reversibly by glycogen phosphorylase, which splits off G1P; the enzyme is inhibited by G6P (Parrou et al., 1997) and UDPG (Tanabe et al., 1987, 1988).

This brief summary illustrates that the metabolic control of trehalose is dynamic and highly dependent on the environmental milieu, and that situation-appropriate amounts of trehalose are critical for normal cellular functioning. The complexity of this dynamics raises the question of how the cell regulates its trehalose cycle and how it organizes and coordinates effective responses to different conditions involving heat and the availability of glucose.

As any other metabolic pathway, the trehalose cycle exhibits distinct structural and mechanistic features at three organizational levels. The first consists of the kinetic conversions between metabolites and captures the stoichiometric distribution of mass through metabolic fluxes. The second level addresses the flow of information, typically in the form of direct inhibition or activation, or in the form of a more indirect repression or induction of the corresponding genes. No flow of material occurs at this level, but there is transduction of information, which may be mediated directly through a conformational change of the modulator or indirectly through a signaling pathway. The third level reflects the operation of the pathway under specific conditions. With the structures of material flow and signal transduction in place, the question here is how particular steps are up- or down-regulated, induced or repressed, in order to achieve an effective, coordinated response to a given stimulus. In the case of trehalose cycling, stimuli of premier interest are heat shock and different degrees of external glucose availability.

The flow of material through the trehalose cycle is more or less dictated by biochemical necessity. Trehalose is a disaccharide, which requires two hexoses for its production. The simple hexosyl group transfer with subsequent dephosphorylation is possibly optimized for functional efficiency, as it was demonstrated for other pathways, such as the pentose phosphate pathway and the Krebs cycle (e.g. Meléndez-Hevia, 1990; Mittenthal et al., 2001). The cell resorts to a different mechanism of trehalose production, using a maltose pathway, only when starved for glucose (Ferreira et al., 1997).

In contrast to the flow of material, the regulatory structure of the trehalose cycle (Fig. 1) is not intuitive and raises non-trivial questions. For instance, what is the significance of glucose repressing the production of T6P and, conversely, what is the significance of T6P inhibiting the main hexokinase that phosphorylates glucose to G6P? What is the advantage of G6P activating the incorporation of UDPG into glycogen and inhibiting the splitting of hexose from glycogen?

The third level of pathway operation (Fig. 2) is even less obvious. As an example, if the cell’s immediate goal is a rapid accumulation of trehalose, why does heat induce the incorporation of UDPG into glycogen, a process that in direct competition with the formation of T6P and trehalose (Stanford Database, 2003)? What is the benefit of not inducing phosphofructokinase or the main hexokinase (Stanford Database, 2003)? If trehalose is needed for protection against heat, what is the significance of heat inducing trehalase, which degrades the valuable disaccharide (De Virgilio et al., 1991a, b, 1993; Neves and François, 1992; Nwaka et al., 1995a,b; Parrou et al., 1997)? The canonical analysis will shed light on these questions.

3. Data

All data were obtained from publicly available sources. Biochemical and physiological information was retrieved from the original literature and through curated websites, especially Brenda (2003) and YPD (Incyte, 2003). This information was used to establish the flow and regulatory organization of the trehalose cycle and to estimate parameter values for the numerical implementation of the canonical model.

Much of the kinetic information needed for setting up the canonical model was obtained from a series of articles with data and kinetic models of glycolysis in yeast, which were described and analysed elsewhere (Galazzo and Bailey, 1990; Schlosser et al., 1994; Curto et al., 1995; Torres et al., 1997). Specific details about the trehalose cycle were incorporated into a subsequent model (Voit and Radivojevitch, 2000) according to the guidelines of BST. Because these earlier models are readily available in the literature, the following sections emphasize only features that are different and of particular regulatory importance.
Table 1
Genes involved in trehalose and glycogen dynamics, along with corresponding enzymes, EC numbers, variable names in the canonical model (1), averaged levels of up-regulation 10–20 min after heat shock, and numbers of mRNA copies.

<table>
<thead>
<tr>
<th>Code</th>
<th>Gene</th>
<th>Enzyme or step</th>
<th>EC number</th>
<th>Variable name</th>
<th>Increase</th>
<th>mRNA copies/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>YPR026W</td>
<td>ATH1</td>
<td>Vacuolar acid α,β-trehalase</td>
<td>3.2.1.28</td>
<td>X19</td>
<td>8.17</td>
<td>0.2</td>
</tr>
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<td>YCL040W</td>
<td>GLK1</td>
<td>Glucokinase</td>
<td>2.7.1.2</td>
<td>X9</td>
<td>39.95</td>
<td>3.70</td>
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<td>GPH1</td>
<td>Glycogen phosphorylase</td>
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<td>X15</td>
<td>111.43</td>
<td>0.6</td>
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<td>YFR015C</td>
<td>GSY1</td>
<td>Glycogen synthase</td>
<td>2.4.1.11</td>
<td>X14</td>
<td>16.22</td>
<td>0.1</td>
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<td>YLR258W</td>
<td>GSY2</td>
<td>Glycogen synthase</td>
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<td>X14</td>
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<td>YKR058W</td>
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<td>Glycogen synthesis initiator</td>
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<td></td>
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<tr>
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<td>Glycogen synthesis inhibitor</td>
<td></td>
<td></td>
<td>1.55</td>
<td></td>
</tr>
<tr>
<td>YOR178C</td>
<td>GAC1</td>
<td>Glic7 regulatory subunit</td>
<td></td>
<td></td>
<td>8.06</td>
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<tr>
<td>YEL011W</td>
<td>GLC3</td>
<td>Glycogen branching enzyme</td>
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<td>X16</td>
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<td></td>
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<tr>
<td>YPR184W</td>
<td></td>
<td>Similar to glycogen debranching</td>
<td></td>
<td></td>
<td>27.47</td>
<td></td>
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<tr>
<td>YFR053C</td>
<td>HXK1</td>
<td>Hexokinase I</td>
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<td>X9</td>
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<td>3.70</td>
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<td>1.56</td>
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<td>HXT1</td>
<td>Hexose transporter (low affinity)</td>
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<td>X8</td>
<td>0.29</td>
<td>11.2</td>
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<td>Hexose transporter (high affinity)</td>
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<td>HXT7</td>
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<td>X8</td>
<td>25.28</td>
<td>9.90</td>
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<td>YJL214W</td>
<td>HXT8</td>
<td>Hexose transporter (putative)</td>
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<td>X9</td>
<td>1.06</td>
<td>0.2</td>
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<tr>
<td>YIL171W</td>
<td>HXT12</td>
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<td></td>
<td>X9</td>
<td>0.91</td>
<td>0.4</td>
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<td>YDR001C</td>
<td>NTH1</td>
<td>Neutral α,β-trehalase</td>
<td>3.2.1.28</td>
<td>X19</td>
<td>11.47</td>
<td>1.5</td>
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<td>NTH2</td>
<td>Neutral α,β-trehalase</td>
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<td>X19</td>
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<td>PFK1</td>
<td>Phosphofructokinase alpha subunit</td>
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<td>Phosphofructokinase beta subunit</td>
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<td>X10</td>
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<td>YBR196C</td>
<td>PGI1</td>
<td>Glucose 6-phosphate isomerase</td>
<td>5.3.1.9</td>
<td>X10</td>
<td>1.62</td>
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<td>PGM1</td>
<td>Phosphoglucomutase, minor isomform</td>
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<td>X12</td>
<td>1.05</td>
<td>1.80</td>
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<td>PGM2</td>
<td>Phosphoglucomutase, major isomorm</td>
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<td>X12</td>
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<td>0.30</td>
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<td>YBR126C</td>
<td>TPS1</td>
<td>α,β-Trehalose 6-phosphate synthase</td>
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<td>X17</td>
<td>12.90</td>
<td>2.8</td>
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<td>TPS2</td>
<td>α,β-Trehalose 6-phosphate phosphatase</td>
<td>3.1.3.12</td>
<td>X18</td>
<td>18.90</td>
<td>0.7</td>
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<td>YMR261C</td>
<td>TPS3</td>
<td>α,β-Trehalose 6-P synthase regulator</td>
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<td></td>
<td>3.32</td>
<td>1</td>
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<td>TSL1</td>
<td>α,β-Trehalose 6-P synthase regulator</td>
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<td>83.29</td>
<td>1.3</td>
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<td>UDP glucose pyrophosphorylase</td>
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<td>19.03</td>
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<td>YNL241C</td>
<td>ZWF1</td>
<td>Glucose 6-phosphate dehydrogenase</td>
<td>1.1.1.49</td>
<td>X11</td>
<td>5.06</td>
<td>1.60</td>
</tr>
</tbody>
</table>

Expression data are weighted raw data from the Stanford Database (2003) (see also Eisen et al., 1998). mRNA copy numbers are taken from Young (2003) (Wodicka et al., 1997; Holstege et al., 1998). Enzymes catalysing the same metabolic step are summarily represented in the model by one variable.

*Value from 1999 Stanford Database (see Voit and Radivojevitch, 2000).

Data on gene expression and patterns of up- or down-regulation after heat shock were extracted from the Stanford yeast database (Eisen et al., 1998; Stanford Database, 2003). Within minutes after heat shock, the cell initiates a coordinated response, in which most genes associated with the trehalose cycle are up-regulated, but to widely varying degrees (Table 1). Notable exceptions are HXK2, which codes for hexokinase II and is responsible for most of the phosphorylation of glucose to G6P under normal conditions, and PFK1/2, which code for phosphofructokinase, the catalyst for the first committed step of glycolysis. These two steps are not up-regulated in response to heat shock (Stanford Database, 2003).

As is to be expected, the expression patterns are dynamic, but it appears that they are more or less stable during a period between 10 and 20 min after heat shock. The levels of up-regulation during this interval were used as estimates for the corresponding changes in enzyme activities. For instance, if the expression data indicated eight-fold up-regulation of a particular gene, the corresponding enzyme activity was increased eight-times. For steps involving several enzymes, the overall increase in activity was computed as average, weighted by the mRNA copy numbers for each contributing gene, as described elsewhere (Voit and Radivojevitch, 2000). The direct extrapolation from gene expression to enzyme activity is clearly a simplification. However, it seems to be a reasonable default assumption, because experimental evidence suggests that many enzymes associated with glucose phosphorylation and heat-shock response are regulated at the level of transcription (Lindquist, 1986; Rodriguez et al., 2001), and that there is close correspondence between gene induction and corresponding changes in the amount of enzyme (e.g. Vuorio et al., 1993; Parrou et al., 1997, 1999).
4. Methods

4.1. Concepts of model design

The main challenge in any modeling effort is the choice of the best-suited model structure. While biochemical tradition often uses Michaelis–Menten rate laws as the basic description of enzyme-catalysed reactions, these functions become unwieldy in larger systems and raise questions about their validity in vivo (cf. Savageau, 1992, 1995; Torres and Voit, 2002). An alternative is the use of power-law rate laws, which form the foundation of BST (Savageau, 1969a,b) and provide an excellent compromise between biological relevance and mathematical tractability. Because BST provides straightforward recipes for setting up model equations from kinetic and regulatory information and for analysing them, this type of mathematical approach is called canonical modeling (Savageau and Voit, 1987; Voit, 1991). BST and canonical modeling have been discussed and reviewed numerous times (e.g. Savageau, 1976, 1992; Voit, 1991, 2000, 2002; Torres and Voit, 2002), which allows a minimized description of the mathematical background and theoretical aspects of the analysis.

In generic terms, canonical models are constructed as follows. Each metabolite that changes over time is represented by a dependent variable, whose concentration or value at every given time point is governed by an ordinary differential equation. This equation relates the dynamic changes in the metabolite to influxes and effluxes and accounts for all constituents of the system that directly influence the fluxes, for instance, as substrates or through inhibition. Exact mathematical formulations for the fluxes are unknown, but a large body of evidence demonstrates that products of power-law functions are often valid and effective representations. This type of S-system formulation within BST may look unfamiliar to the uninitiated, but it has strong mathematical justification and a proven track record in biology and, especially, in metabolic pathway analysis and gene regulation (e.g. Savageau, 1976; Voit, 2000).

Thus, a given flux is formulated as a product of power-law functions and contains as variables all metabolites and modulators that directly affect this flux. This information is immediately available from biochemical information about the stoichiometry and regulation of the pathway. For instance, to assemble the production term of T6P according to Fig. 1, G6P and UDPG are included as substrates and glucose is included as repressor; the catalysing enzyme also becomes a factor in the term. The degradation term of T6P is constructed in the same manner. It simply consists of T6P as substrate and the catalysing enzyme T6P phosphatase. Kinetic order parameters are included as exponents of all variables involved in a term. They quantify the strengths of the influences that the variables have on a particular flux and are analogous to kinetic orders in elemental chemical reactions, but may be non-integer in BST. For instance, as a substrate for T6P synthase, UDPG will have a positive kinetic order, whereas glucose will be assigned a negative kinetic order, because glucose represses this catalytic step. Also analogous to an elemental chemical reaction, a rate constant (multiplier) quantifies the overall magnitude of each flux. Various methods are available for estimating numerical values of these parameters from experimental data (Voit, 2000, Chapter 5).

4.2. A canonical model of the trehalose cycle

The mathematical structure of the trehalose model is deduced directly from Fig. 1 and implemented as a numerical model with parameter values and information from the literature that were discussed elsewhere (Galazzo and Bailey, 1990; Schlosser et al., 1994; Curto et al., 1995; Torres et al., 1997; Voit and Radivoyevitch, 2000). The resulting S-system equations, which are used for all analyses, are

\[
\begin{align*}
\text{Glucose:} & \quad \dot{X}_1 = 31.912X_0^{0.968}X_2^{-0.194}X_3^{0.00968}X_4^{0.968}X_5^{0.0323}X_6^{1.19} - 89.935X_1^{0.75}X_6^{0.4}X_9 \\
\text{G6P:} & \quad \dot{X}_2 = 142.72X_0^{0.517}X_2^{-0.179}X_3^{0.183}X_6^{-0.276}X_7^{0.689}X_9^{1.311} - 30.12X_1^{0.00333}X_2^{0.575}X_3^{0.17}X_4^{0.00333}X_5^{0.5111}X_6^{0.0667}X_7^{1.411}X_9^{0.0111} \\
\text{G1P:} & \quad \dot{X}_3 = 7.8819X_2^{0.394}X_3^{-0.392}X_4^{-0.010}X_5^{0.0128}X_6^{0.049}X_7^{0.0513} - 76.434X_2^{-0.412}X_3^{0.593}X_4^{0.718}X_5^{0.180}X_6^{0.103}X_7^{0.157}X_9^{0.157} \\
\text{UDPG:} & \quad \dot{X}_4 = 11.070X_2^{0.5}X_3^{0.13} - 3.4556X_4^{-0.0429}X_5^{0.214}X_6^{0.386}X_7^{0.857}X_9^{0.143} \\
\text{Glycogen:} & \quad \dot{X}_5 = 11.060X_2^{0.040}X_3^{0.320}X_4^{0.160}X_5^{0.600}X_6^{0.400} - 4.9290X_2^{-0.04}X_3^{-0.04}X_4^{-0.25}X_5^{0.16}X_6^{0.800}X_7^{0.153}X_9^{0.153} \\
\text{T6P:} & \quad \dot{X}_6 = 0.19424X_1^{-0.300}X_2^{0.300}X_3^{0.300}X_4^{-0.300}X_5^{0.17} - 1.0939X_6^{0.200}X_9^{0.18} \\
\text{Trehalose:} & \quad \dot{X}_7 = 1.0939X_6^{0.200}X_8^{1.2288}X_9^{0.300}X_9^{1.919}.
\end{align*}
\]
In these differential equations, $X_0$ is the external glucose concentration, which is defined as 1 under normal conditions and raised or lowered to reflect abundant or minimal glucose availability. The dependent variables $X_1, \ldots, X_7$ are characterized in Table 2 with their steady-state concentrations, which were also used as initial values in perturbation experiments and heat-shock simulations, and their steady-state fluxes. In addition to the dependent variables, the equations contain groups of transporters and enzymes that are coded as independent variables $X_8, \ldots, X_{19}$ (Table 3). Their nominal values at baseline are defined as 1, but will later be reset to reflect heat-shock levels. The indices $f$ and $r$ refer to forward and reverse directions of reversible reactions.

In addition to the canonical S-system model in Eq. (1), the corresponding generalized mass action (GMA) model was constructed as a quality check. GMA and S-systems are two types of canonical BST models that both employ power-law representations but implement them in a slightly different mathematical fashion; the literature has extensively discussed their respective advantages and drawbacks (e.g. Savageau et al., 1987a; Voit, 2000). Both types of models yielded results that were qualitatively equivalent and quantitatively similar. Only the results of the S-system analysis are shown. All analyses were executed with the freeware PLAS® (Voit, 2000; Ferreira, 2000); a corresponding MATLAB toolbox is being tested in our laboratory.

### 4.3. Controlled comparisons

BST offers an elegant method for evaluating the effects of a particular structural feature: the MCMC (e.g. Savageau, 1985; Irvine, 1991; Alves and Savageau, 2000). In this method, the investigated pathway is compared with an alternative, hypothetical pathway that differs in just one particular regulatory or operational feature. The key strength of controlled comparisons is that the remainder of the network is intact, so that changes in responses can be attributed uniquely to the feature of interest. The comparative approach also has the advantage of the cancellation of many uncertain quantities if they affect the investigated and the alternative pathway in the same fashion.

As a typical, generic example, consider feedback inhibition by product $X_n$ on the synthesis of an intermediate $X_1$. Suppose the synthesis of $X_1$ is represented by the power-law term $x_1x_0^{g_1n}x_k^{g_kn}$, where $X_0$ is the substrate for the process and $X_k$ is some modulator. The inhibition by product $X_n$ is represented by the kinetic order $g_{1n}$, which is a real number with negative value, indicating inhibition rather than activation. According to MCMC, a system A with inhibition is compared to an otherwise equivalent system B without inhibition in the following manner. First, system B is characterized by the lack of inhibition: $g_{1n} = 0$. Because $X_n^{g_{1n}} = X_n^0 = 1$, this setting effectively causes elimination of $X_n$ as a modulator of the synthesis of $X_1$. Second, the two systems should be equivalent to

---

### Table 2
**Dependent variables of the canonical model (1) of the trehalose cycle**

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Variable name</th>
<th>Steady-state concentration (mM)</th>
<th>Steady-state flux (mM min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>$X_1$</td>
<td>0.03</td>
<td>31</td>
</tr>
<tr>
<td>Glucose-6-phosphate</td>
<td>$X_2$</td>
<td>1</td>
<td>45</td>
</tr>
<tr>
<td>Glucose-1-phosphate</td>
<td>$X_3$</td>
<td>0.1</td>
<td>19.5</td>
</tr>
<tr>
<td>Uridine diphosphate glucose</td>
<td>$X_4$</td>
<td>0.7</td>
<td>3.5</td>
</tr>
<tr>
<td>Glycogen</td>
<td>$X_5$</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Trehalose 6-phosphate</td>
<td>$X_6$</td>
<td>0.02</td>
<td>0.5</td>
</tr>
<tr>
<td>Trehalose</td>
<td>$X_7$</td>
<td>0.05</td>
<td>0.5</td>
</tr>
</tbody>
</table>

### Table 3
**Enzyme activities and transport steps that were used in the canonical model (1), along with increases induced by heat shock**

<table>
<thead>
<tr>
<th>Catalytic or transport step</th>
<th>Variable name</th>
<th>EC number</th>
<th>Heat-induced fold increase in activity used in the model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose transport into cell</td>
<td>$X_8$</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Hexokinase</td>
<td>$X_9$</td>
<td>2.7.1.1</td>
<td>8</td>
</tr>
<tr>
<td>Glucokinase</td>
<td></td>
<td>2.7.1.2</td>
<td></td>
</tr>
<tr>
<td>Phosphofructokinase</td>
<td>$X_{10}$</td>
<td>2.7.1.11</td>
<td>1</td>
</tr>
<tr>
<td>G6P dehydrogenase</td>
<td>$X_{11}$</td>
<td>1.1.1.49</td>
<td>6</td>
</tr>
<tr>
<td>Phosphoglucosemutase</td>
<td>$X_{12}$</td>
<td>5.4.2.2</td>
<td>16</td>
</tr>
<tr>
<td>UDPG pyrophosphorylase</td>
<td>$X_{13}$</td>
<td>2.7.7.9</td>
<td>16</td>
</tr>
<tr>
<td>Glycogen synthase</td>
<td>$X_{14}$</td>
<td>2.4.1.11</td>
<td>16</td>
</tr>
<tr>
<td>Glycogen phosphorylase</td>
<td>$X_{15}$</td>
<td>2.4.1.1</td>
<td>50</td>
</tr>
<tr>
<td>Glycogen use</td>
<td>$X_{16}$</td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>α-α-T6P synthase</td>
<td>$X_{17}$</td>
<td>2.4.1.15</td>
<td>12</td>
</tr>
<tr>
<td>α-α-T6P phosphatase</td>
<td>$X_{18}$</td>
<td>3.1.3.12</td>
<td>18</td>
</tr>
<tr>
<td>Trehalase</td>
<td>$X_{19}$</td>
<td>3.2.1.28</td>
<td>6</td>
</tr>
</tbody>
</table>
the outside observer. For fair comparisons, their steady states should be the same and have the value that was experimentally determined. To enforce this “external equivalence,” the synthesis term of $X_1$ in systems A and B must have the same numerical value. This is accomplished by adjusting the rate constant $z_{1B}$ of the term in system B, such that $z_{1B}X_0^{g_1}X_1^{a_1} = z_{1A}X_0^{g_1}X_1^{a_1}X_n^{r}$. With the adjustments in $g_{1s}$ and $z_{1B}$, the systems behave exactly the same, as long as $X_n$ is not involved. In particular, the role of the modulator $X_k$ is untouched. At the same time, a comparison of stability, sensitivities, robustness, and dynamic responses reveals the specific and unique effects that the inhibition by $X_n$ has on the pathway.

5. Results and discussion

As a direct consequence of the chosen type of analysis, most results are comparative. Some compare the responses of the canonical model with those observed in the living cell, others compare the outputs of two alternative models, one of which represents the actual pathway structure, while the other one represents a similar pathway that differs from the actual pathway in one distinct feature, such as a regulatory signal. To facilitate recognition of which results refer to which system, particular results will be preceded with (Obs) for an “Observation” in the living cell, (YM) for the “Yeast Model” with the numerical specifications given before, or (AM) for any type of hypothesized “Alternative Model.”

5.1. Regulation under normal conditions

The trehalose cycle must be regulated to respond appropriately to combinations of different conditions. If glucose availability in the medium is low, most of it must be used for glycolysis and maybe the pentose cycle. If glucose is present in abundance, much of it should be stored as glycogen, which is an energetically efficient storage metabolite. If the temperature increases, however, the first responsibility of the cycle is to produce sufficient amounts of trehalose for protection. This first part of the comparative model analysis focuses on trehalose production in wild type and mutants under normal physiological conditions, while the second part highlights regulation following heat shock.

5.1.1. Baseline analysis

Obs: Bonini et al. (1995) observed that ATP, free phosphate, trehalose, glycogen, and key glycolytic metabolites decrease within 15 min after depletion of the external carbon source. Although these measurements were made in Neurospora crassa, the authors stated that similar results were to be expected for yeast.

YM: The model with parameter specifications obtained from the literature (Eq. (1)) exhibits similar dynamic features. If the input of external glucose is abruptly terminated, trehalose, glycogen, and the flux into glycolysis decrease within minutes. The time to depletion is somewhat shorter than observed, because the simulation terminated glucose input immediately and completely, whereas external glucose was taken up gradually in the experiments of Bonini and co-workers. Gradual depletion of the external glucose supply leads to decreases in model metabolites consistent with observations. Eigenvalue analysis (Savageau, 1975) proves the steady state of Eq. (1) to be stable. A complete analysis of parameter sensitivities and gains (Savageau, 1972) further demonstrates that the model (Eq. (1)) is robust and that moderate changes in conditions or model structure do not much affect the responses of the model. For instance, the percent change in a steady-state value that is caused by a 1% change in an independent variable is measured in BST as a logarithmic gain. Out of 105 logarithmic gains in the trehalose model, 71.3% are between 0 and 1 in magnitude, indicating that a perturbation is attenuated, 20% are between 1 and 2, indicating slight amplification, 6.7% are between 2 and 3, and 2% are between 3 and 4. No gain is larger than 3.4. Thus, small changes in enzymes or transporters are readily tolerated. Sensitivities with respect to parameters are similarly small. This suggests that inaccuracies in the estimation of parameter values are not overly influential. Overall, the model has no apparent structural faults.

Obs: Both glycogen and trehalose serve as storage carbohydrates. Under conditions of glucose abundance, they accumulate dramatically and can make up more than 20% of the dry cell mass (e.g. Lillie and Pringle, 1980; Parrou et al., 1997). Although they have a similar role in this respect, glycogen is energetically superior (Wiemken, 1990), which renders its preferential production advantageous under normal temperature conditions. Consistent with its role as carbohydrate storage, glycogen is consumed to release sugar phosphates if external glucose is in short supply. In contrast to glycogen, trehalose plays the additional important role of a stress protectant. Therefore, it benefits the cell to generate, accumulate and retain trehalose rather than glycogen, if glucose is scarce and the cell is stressed, and to shift to a different energy source before using up all of its trehalose.

YM: If the input of glucose is permanently doubled, the glycolytic flux in the model increases by 80% and trehalose by 40%, but the glycogen pool grows five-fold, clearly confirming preferred glycogen storage under opportune glucose conditions. Conversely, if the model pathway experiences glucose shortage, glycogen production is strongly reduced and the glycogen pool decreases dramatically. For instance, at 25% of the normal
external glucose, the glycogen pool decreases to 5% of its original size, while trehalose only decreases to about half the original pool size.

5.1.2. Mutants

Mutations are represented in the model through a strong or total reduction in enzyme activity. The model is solved and the responses are compared to observations obtained from the literature. As is demonstrated below, the model captures mutations in key enzymes of the trehalose cycle and their observed ramifications quite well.

5.1.2.1. TPS1. Obs: A mutation in this gene leads to impaired activity of T6P synthase (Bell et al., 1992, 1998; Vuorio et al., 1993). It results in the cell’s failure to produce T6P and trehalose (van Dijck et al., 1995) and causes strongly compromised thermo-tolerance (Argüelles, 1994; De Virgilio et al., 1994; Singer and Lindquist, 1998b). As a secondary effect, TPS1 deletion mutations also destabilize the trehalose synthesis complex, which results in decreased T6P phosphatase activity (Reinders et al., 1997).

TPS1 deletion mutants are unable to grow on glucose substrate (De Virgilio et al., 1991a), because they do not properly control glucose flux into glycolysis. Ernandes et al. (1998) observed a resultant accumulation of cytoplasmic glucose. Other consequences of the inadequate glucose control are hyperaccumulation of sugar phosphates (Neves et al., 1995) and glycogen (Cannon et al., 1994), as well as a rapid depletion of ATP and free phosphate (Bonini et al., 1995; van Vaeck et al., 2001). The combination of high glucose and a short supply of ATP probably leads to a bottleneck at the glyceraldehyde-3-phosphate dehydrogenase step during glycolysis, because the elevated glucose level triggers higher hexokinase activity, which consumes free phosphate (Thevelein and Hohmann, 1995). This hypothesis is consistent with the observation that ethanol production in TPS1 mutants is reduced (Noubhani et al., 2000).

YM: The TPS1 mutation is readily implemented in the model as reduced activity of the T6P phosphatase step. As expected, this alteration results in a greatly expanded T6P pool and in a trehalose concentration that corresponds to just 1% of that in the model wild type. G6P and the glycolytic flux are slightly reduced, but the internal glucose concentration is strongly increased, which is probably due to the fact that the elevated T6P exerts stronger inhibition upon hexokinase II, thereby reducing its activity. This mechanism affirms the role of the trehalose cycle in the regulation of glucose metabolism (Bonini et al., 2000).

5.1.2.2. TPS2. Obs: A mutation in TPS2 leads to a strong reduction in the activity of T6P phosphatase (Reinders et al., 1997), which leads to a significant and toxic accumulation of T6P (Sur et al., 1994; Thevelein and Hohmann, 1995) and depletion of the trehalose pool (De Virgilio et al., 1993, 1994; Bell et al., 1998; van Vaeck et al., 2001). This becomes a problem under heat shock, where TPS2 mutants show increased sensitivity and fail to grow (Sur et al., 1994). As a secondary effect of the mutation, hexokinase activity and G6P accumulation are reduced (Noubhani et al., 2000). TPS2 deletion mutants exhibit strongly reduced viability presumably because of phosphate sequestration and consequent intracellular acidification (Thevelein and Hohmann, 1995). Analogous to TPS1 deletion mutants, deletion in TPS2 leads to destabilization of the trehalose synthesis complex and diminished T6P synthase activity (Reinders et al., 1997). However, in this case the effect is apparently not as strong, because free tps1 protein is able to produce T6P without the involvement of the TPS/TSL complex (Reinders et al., 1997; Noubhani et al., 2000).

YM: The TPS2 mutation is readily implemented in the model as reduced activity of the T6P phosphatase step. As expected, this alteration results in a greatly expanded T6P pool and in a trehalose concentration that corresponds to just 1% of that in the model wild type. G6P and the glycolytic flux are slightly reduced, but the internal glucose concentration is strongly increased, which is probably due to the fact that the elevated T6P exerts stronger inhibition upon hexokinase II, thereby reducing its activity. This mechanism affirms the role of the trehalose cycle in the regulation of glucose metabolism (Bonini et al., 2000).

5.1.2.3. TPS1/TPS2. Obs: TPS1/TPS2 double mutants do not accumulate trehalose (Bell et al., 1998; Benaroudj et al., 2001). As in the case of a TPS1 deletion, ethanol production is decreased (Noubhani et al., 2000). In contrast to TPS2 mutations, TPS1/TPS2 double mutant do not show reductions in hexokinase activity (Noubhani et al., 2000).

YM: As one would expect, the model pathway does not produce trehalose. Interestingly, the model also indirectly confirms the lower ethanol production. A direct confirmation is not possible, because ethanol is not modeled per se. However, UDPG and glycogen are elevated in the model, and this leads to a slight reduction in the concentration of G6P and a subsequent decrease in glycolytic flux, which would result in a lower level of ethanol, were it included in the model. Of note is that
the \textit{TPS1/TPS2} double mutant in the model shows a normal level of internal glucose, which is in contrast to the single \textit{TPS1} mutant. An explanation is that the double mutant exhibits an essentially normal T6P concentration, because production and degradation of T6P are both strongly reduced. Thus, the flux through the T6P pool is very low, but the concentration remains close to normal. As a consequence, the inhibition of hexokinase II is similar to that in wild type, whereas it is removed in the single \textit{TPS1} mutant, thereby allowing faster conversion of glucose into G6P.

\subsection*{5.1.2.4. \textit{TPS1/HXK2}}
\textbf{Obs}: A mutation in \textit{TPS1} alone leads to a decrease in T6P, the consequence of which is diminished inhibition of hexokinase II. In \textit{TPS1/HXK2} double mutants, the absence of inhibition is immaterial, since hexokinase II is not expressed. Indeed, Blázquez and Gancedo (1994) determined the glycolytic flux in \textit{TPS1/HXK2} double mutants to be normal, which may be due to the fact that low activities in hexokinase II are compensated by hexokinase I and glucokinase (Hohmann et al., 1993; Boy-Marcotte et al., 1999; Bonini et al., 2000; Rodriguez et al., 2001). The normal glycolytic functioning of the \textit{TPS1/HXK2} double mutant is corroborated by the observation that deletion of \textit{HXK2} restores growth on glucose in \textit{TPS1} mutants (Hohmann et al., 1993; van Dijck et al., 1995; van Vaek et al., 2001).

\textbf{YM}: The \textit{TPS1/HXK2} double mutant is represented in the model through two alterations. First, the lack of \textit{TPS1} is captured by a strong reduction or total elimination of the activity of T6P, and secondly, the conversion of glucose to G6P is made independent of the inhibition by T6P. The model result agrees with experimental observations: no T6P or trehalose is produced, the glycolytic flux is normal, and glucose, G6P and G1P are very close to wild-type levels.

\subsection*{5.1.2.5. \textit{NTH1}}
\textbf{Obs}: Deletion of this gene leads to a lack of neutral trehalase; double mutants \textit{NTH1/NTH2} and \textit{NTH1/ATH1} show similar characteristics (Shima et al., 1999). The immediate consequence of impaired trehalase activity in vivo is an increased level of trehalose, especially under heat-shock conditions (van Dijck et al., 1995; Parrou et al., 1997, 1999; Iwahashi et al., 2000; Zähringer et al., 2000). Since the degradation of trehalose is compromised in these mutants, their recovery from heat shock is slowed down (De Virgilio et al., 1991b, 1994). Also of note is that overproduction of neutral trehalase results in a rapid depletion of trehalose, which can be lethal (Espinet et al., 1995).

\textbf{YM}: Consistent with observations, reduction in trehalase activity in the model leads to a drastic accumulation in trehalose, which is even more strongly pronounced under heat shock, when much more trehalose is produced. Also as observed, overproduction of trehalase depletes the trehalose pool, with all negative consequences, especially after heat shock.

\subsection*{5.1.3. Summary of baseline and mutant experiments}
The baseline analysis detected no obvious flaws in the model; it affirmed a steady state consistent with information from the literature, was stable, and exhibited gains and sensitivities that attest sufficient robustness. A direct quantitative confirmation of intermediate metabolite levels in the model was not feasible because of lacking experimental data, but some quantitative results are available for heat-shock conditions; they are modeled well, as is discussed in a later section. The model seems to capture accurately the consequences of deletions and changes in enzyme activities for which observations are available. While no mathematical proof, the good correspondence between observations and model predictions lends credence to the model structure and implementation and allows us to evaluate the roles of the different regulatory signals.

\subsection*{5.1.4. Regulation of the trehalose cycle}
To deduce a rational, mechanistic explanation for each regulatory signal of the trehalose cycle, we use the MCMC. In particular, we compare the model of the observed pathway, which exhibits a given regulatory signal \textit{YM}, to an alternative, otherwise equivalent pathway model without this signal \textit{AM}. The results, summarized in Table 4, collectively suggest that the various regulatory signals cooperate synergistically, thereby achieving two goals: They aid in the control of the internal glucose pool and facilitate efficient channeling of glucose toward glycolysis, glycogen or trehalose, depending on physiological demands. If any one of these signals is missing, the model cell does not seem to run into disastrous difficulties, but each signal offers a slight advantage over an unregulated system, and taken together, the regulatory structure is better suited to handle perturbations than an alternative, less regulated system. The concentration of G6P and the glycolytic flux are largely unaffected by the presence or absence of these regulatory signals. This is not too surprising, because the trehalose flux is much smaller than the glycolytic flux.

\subsubsection*{5.1.4.1. Repression of T6P synthase by glucose}
In comparison to the model wild-type \textit{YM}, the model cell without this regulatory signal \textit{AM} does not process abundant external glucose well. Carbohydrate storage in the form of glycogen is reduced, and some of the excess sugar is instead channeled toward trehalose. The remaining glucose accumulates, because the lacking repression causes an elevation in T6P level, which in turn leads to stronger inhibition of hexokinase II and reduced phosphorylation of glucose to G6P. The
glycolytic flux is essentially unaffected by the lack of repression.

**Obs:** The accumulation of glucose is generally undesirable, because glucose induces or represses a slew of pathways and can be toxic in elevated concentrations (Entian et al., 1984; Hottiger et al., 1987). Also, the increased T6P level is disadvantageous because of toxicity (Sur et al., 1994; Thevelein and Hohmann, 1995). It is noted that this regulatory signal is realized in vivo as repression of gene expression, which is more indirect and considerably slower than competitive or allosteric enzyme inhibition. The dynamic consequences of this strategy are still to be evaluated.

5.1.4.2. Induction of T6P synthase by G6P and UDPG. **Obs:** The specific activity of T6P synthase increases hyperbolically with the availability of its two substrates, G6P and UDPG (Lapp et al., 1971; Vandercammen et al., 1989). **AM:** Removal of either one of these inductive effects, which are implemented in the model by lowering the corresponding kinetic orders and adjusting the rate constant to restore the original steady-state flux, causes less T6P and trehalose to be produced. As a consequence, the internal glucose level is somewhat lower and glycogen is slightly increased. The two inductions act synergistically. At normal temperature, the differences are less significant, but under heat-shock conditions and with normal glucose availability, 40% less T6P and 30% less trehalose is produced in comparison to the regulated system **YM**. The differences are even more pronounced if glucose is abundant.**

5.1.4.3. Inhibition of hexokinase II by T6P. **Obs:** This inhibition is apparently important in vivo, because the deletion of T6P, which removes this inhibition, leads to a lethal accumulation of fructose 1,6-diphosphate in many strains of *S. cerevisiae* (Thevelein and Hohmann, 1995). Bonini et al. (2000) concluded from specific inhibition experiments that T6P alone is not responsible for the down-regulation of hexokinase and that some of the inhibition may derive from free tsp1 protein (see also Noubhani et al., 2000).

**AM:** Removal of this inhibition leads again to a slightly inferior channeling of glucose, resulting in less glycogen and more T6P and trehalose. The difference under normal operating conditions is small, but under glucose-rich heat conditions, the model wild-type **YM** stores more glycogen, while still maintaining sufficient trehalose levels. Fructose 1,6-diphosphate is not represented in the model, so that this mechanism cannot be studied directly.

5.1.4.4. Effect of G6P and UDPG on glycogen dynamics. **Obs:** G6P activates glycogen synthase (Peng et al., 1990). **AM:** Without activation of glycogen synthase, the model pathway produces considerably less glycogen and instead produces more T6P and trehalose than the model wild-type **YM**. For instance, if external glucose is doubled in the medium, the wild-type model **YM** stores 50% more glycogen than the unregulated model **AM**. The concentration of G6P and the glycolytic flux are essentially equivalent in both models.

**Obs:** G6P and UDPG both inhibit glycogen phosphorylase, which converts G1P into glycogen and vice versa (Tanabe et al., 1987, 1988). A comparison between the model wild-type **YM** and an otherwise equivalent model **AM** without these two inhibitions does not exhibit much difference. However, if one takes into account that the phosphate level decreases to half its baseline level under heat conditions (Winkler et al., 1991; Bonini et al., 2000; van Vaeck et al., 2001), especially under glucose-poor conditions, one should expect a reduced reverse glycogen phosphorylase reaction, which splits hexoses off the glycogen molecule and combines them with phosphate to form G1P. With this alteration, the model with regulation **YM** performs

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**Table 4**

<table>
<thead>
<tr>
<th>Regulatory signal</th>
<th>Condition</th>
<th>Effect on Glucose</th>
<th>Effect on Glycogen storage</th>
<th>Effect on T6P</th>
<th>Effect on Trehalose</th>
<th>Effect on Glycolytic flux</th>
</tr>
</thead>
<tbody>
<tr>
<td>No repression of T6P synthase by glucose</td>
<td>HG&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Accumulates</td>
<td>Reduced&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Elevated</td>
<td>Elevated</td>
<td>c</td>
</tr>
<tr>
<td>No induction of T6P synthase by G6P, UDPG</td>
<td>HS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No inhibition of hexokinase</td>
<td>HG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No regulation of glycogen dynamics</td>
<td>HG, LG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No regulation at All</td>
<td>HG</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>LG</td>
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<tr>
<td></td>
<td>NG, HS</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>LG, HS</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>HG = high glucose, NG = normal glucose, LG = low glucose, HS = heat shock.
<sup>b</sup>All effects in relation to wild-type model **YM**.
<sup>c</sup>Empty boxes indicate no or unremarkable change.
better than the equivalent system without regulation $AM$ under glucose-poor, phosphate-poor conditions. For example, the regulated model pathway generates 25% more trehalose than the unregulated model.

5.1.4.5. Pathway without any of the observed regulatory signals. $YM$: The results in the previous sections suggest that every signal contributes in a small way to the coordinated regulation of the trehalose cycle. Even though each contribution in itself is modest and may seem insignificant, the synergism between the regulatory signals has quite dramatic consequences. The power of this synergism can be assessed with the canonical model by eliminating all feedback and feedforward signals simultaneously.

The completely unregulated model $AM$ performs much worse than the wild-type model $YM$. Under normal baseline conditions with abundant external glucose, $AM$ is not all that different, except that over 10 times as much T6P is being generated. This side effect is undesirable, because T6P is toxic and has a strong effect on the control of influx glucose and free phosphates (Sur et al., 1994; Thevelein and Hohmann, 1995). If external glucose is in short supply (e.g. 20% of the baseline concentration), the deficiencies of $AM$ become more obvious: The glycolytic flux is reduced to about half in comparison with the wild-type model $YM$, and glycogen and trehalose are reduced to levels between 1% and 2%. These effects are exacerbated after heat shock: T6P is increased 25-fold under normal glucose conditions $AM$, and if glucose is in demand, the unregulated model $AM$ generates only between 5% and 20% of the trehalose produced by $YM$, while the glycolytic flux is similar or slightly lower. Thus, synergism between the regulatory signals leads to a pathway design that is superior to an otherwise equivalent yet unregulated pathway.

5.2. Responses to heat shock

Heat shock was modeled with the same model (Eq. (1)), in which, however, the enzyme activities were altered as suggested by the data in Table 1, which shows the degrees of up-regulation and the mRNA copy numbers for each enzymatic and transport step. The resulting alterations in enzyme activities, as they were implemented in the model, are presented in Table 3 and visualized in Fig. 2.

$YM$: Numerical analysis showed that the model with heat-shock alterations according to Table 3 has a stable steady state. Furthermore, no scenario with alternative activity patterns was detected where the steady state became unstable. All model implementations were furthermore found to be robust, exhibiting small or moderate sensitivities and gains, which indicate that normal fluctuations in the cellular milieu are easily tolerated. The dynamics of all alternative models seemed reasonable, for instance, in response to changes in external glucose.

5.2.1. Biochemical consequences of heat shock

Obs: The most dramatic shift in metabolites after heat shock is the accumulation of trehalose. Winkler et al. (1991) described a 25-fold increase, and Hottiger et al. (1987, 1994) found trehalose to increase from trace concentrations to very large quantities up to 1 g/g protein or 500 mM. $YM$: In the model, the trehalose concentration increases about 100-fold. This level strongly depends on the degree of trehalose degradation through trehalase. For instance, if trehalase is induced 50% more strongly, the rise in trehalose concentration is only about 25-fold.

Obs: Parrou et al. (1997, Fig. 3a) measured an increase in glycogen of about five-fold. Glycogen is a somewhat peculiar carbohydrate in that the number of molecules does not necessarily grow if more substrate G1P is available. Rather, hexoses are attached to branches of existing glycogen molecules, thereby making the glycogen molecules larger in size but not always in number. $YM$: The model does not allow for glycogen molecules of different sizes, and the glycogen variable may be imagined as a pool of hexoses that grows or decreases directly with the addition or removal of sugar molecules. With the settings of the heat-shock model, the glycogenic hexose pool increases about 50-fold under normal external glucose conditions and reaches correspondingly higher levels in glucose-rich media.

Obs: Neves and François (1992) observed a several-fold increase in intracellular glucose. $YM$: Cytoplasmic glucose in the model increases by about 50% if the external glucose concentration is set at the normal default that Curto et al. (1995) used to parameterize the original model. If the external concentration is doubled, the internal glucose concentration rises about four-fold.

Obs: Winkler et al. (1991) and Neves and François (1992) used experimental conditions that were apparently very similar. Yet, while many of their results support each other, there is a qualitative difference in the observed level of G6P. Winkler and collaborators measured an 11-fold increase, whereas Neves and François reported a three-fold decrease. The graphs in the article of Grba et al. (1979) suggest that G6P remains the same at different temperatures. In N. crassa conidiospore germlings, G6P rises initially for about 20 min, but then returns to its baseline level (Bonini et al., 2000). The differences could potentially be caused by experimental variation, but the yeast articles are otherwise consistent in many aspects. For instance, both Winkler et al. and Neves and François report a several-fold increase in UDPG. $YM$: In the model, G6P rises five-fold and UDPG about three-fold. With higher glucose concentrations in the medium, these values...
increase correspondingly. For instance, for twice the external concentration, G6P and UDPG rise 12 and 4.5-fold, respectively. These results are in line with those of Winkler and collaborators. Under conditions of reduced glucose availability (20% of normal concentration in medium), G6P remains at the baseline, which may reflect the observations of Grba and co-workers. At even lower external concentrations, G6P decreases below baseline, but so do all other metabolites of the trehalose cycle. Thus, without other considerations, the model does not support the results of Neves and François, which suggest simultaneously decreased G6P but increased internal glucose and UDPG.

**Obs:** Closer scrutiny of Neves and François’ article shows that these authors did not find any of the hexokinases induced by heat, which is in contrast to the otherwise observed strong up-regulation in the corresponding genes HXK1 and GLK1 (Stanford Database, 2003) and may indicate a difference in strain. **YM:** Modeling this variance in the hexokinase step, G6P decreases under glucose-poor conditions, but so do internal glucose and UDPG. However, if the activity of UDPG pyrophosphorylase is also increased, the situation changes, and the model generates results in line with Neves and François: intracellular glucose rises, UDPG increases 10-fold, glycogen five-fold, trehalose 40-fold, yet G6P drops to half its baseline value. It is impossible to say whether the strain used by Neves and François had such characteristics, but it is interesting that slightly different patterns of gene expression may yield physiological responses that are qualitatively different than those observed, and that these and other hypothesized patterns can be easily explored with a canonical model.

### 5.2.2. Comparison of results between baseline heat-shock model (YM) and alternatives (AM)

Controlled mathematical comparisons were executed with the baseline heat-shock model (Eq. (1) and Table 3) and with corresponding alternative models. The results are summarized in Table 5 and discussed below in detail.

#### 5.2.2.1. Glucose transport and kinase step.

**Obs:** The transport of glucose into the cell is rate determining for the entire pathway in vivo (Aon et al., 1995). Furthermore, the kinase step provides the means of making glucose available in the form of the key metabolite G6P. The observed gene expression profile (Stanford Database, 2003) indicates co-regulation in the set of transporters and kinases, which supports the hypothesis that glucose transport into the cell and phosphorylation are coupled processes (Franzusoff and Chirillo, 1982). **YM:** The degree of metabolic response of the model is strongly affected by glucose input. While the various metabolite concentrations do not scale linearly with changes in glucose input, the entire system expands or contracts monotonically in mass with the availability of external glucose. **AM:** Simulations of different patterns of enzyme activities demonstrate clearly that the transport step and the kinase activity need to be up-regulated to a similar degree; otherwise internal glucose accumulates or is depleted over time. For instance, if the kinase activity is left at baseline, but transport is

<table>
<thead>
<tr>
<th>Enzyme/transport step</th>
<th>Much lower up-regulation</th>
<th>Much higher up-regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transport/kinase ratio</td>
<td>Glucose is depleted</td>
<td>Glucose accumulates</td>
</tr>
<tr>
<td>Phosphofructokinase</td>
<td>Glucose accumulates</td>
<td>Strongly reduced trehalose</td>
</tr>
<tr>
<td></td>
<td>Elevated trehalose</td>
<td>Strongly reduced glycogen</td>
</tr>
<tr>
<td></td>
<td>Strongly reduced glycolytic flux</td>
<td>Increased glycolytic flux</td>
</tr>
<tr>
<td>G6P dehydrogenase</td>
<td>Reduced NADPH</td>
<td>Increased NADPH</td>
</tr>
<tr>
<td>Phosphoglucomutase</td>
<td>Strongly reduced trehalose</td>
<td>Modestly increased trehalose</td>
</tr>
<tr>
<td></td>
<td>Strongly reduced glycogen</td>
<td>Modestly increased glycogen</td>
</tr>
<tr>
<td>UDPG pyrophosphorylase</td>
<td>Strongly reduced glucose</td>
<td>Modestly increased trehalose</td>
</tr>
<tr>
<td></td>
<td>Strongly reduced trehalose</td>
<td>Modestly increased glycogen</td>
</tr>
<tr>
<td></td>
<td>Strongly reduced glycogen</td>
<td>Slightly reduced glycolytic flux</td>
</tr>
<tr>
<td>Glycogen synthase</td>
<td>Strongly reduced glycogen</td>
<td>Insignificant changes</td>
</tr>
<tr>
<td></td>
<td>Huge increase in glucose, T6P, and trehalose</td>
<td></td>
</tr>
<tr>
<td>Glycogen phosphorylase</td>
<td>Strongly reduced glycogen, increased glycolytic flux</td>
<td>Insignificant changes</td>
</tr>
<tr>
<td>Trehalose production</td>
<td>Strongly reduced trehalose</td>
<td>Strongly increased trehalose</td>
</tr>
<tr>
<td>Trehalase</td>
<td>Strongly increased trehalose, slow recovery from heat shock</td>
<td>Strongly reduced trehalose</td>
</tr>
</tbody>
</table>
increased as observed, intracellular glucose accumulates five-fold, and only a quarter of trehalose is produced in comparison with YM.

5.2.2.2. Phosphofructokinase. Obs: In contrast to all other steps of the trehalose system, with the exception of HXXK2, the phosphofructokinase genes are not up-regulated at all under heat-shock conditions (Stanford Database, 2003). In N. crassa conidiospore germlings, this step is inhibited, which assists in carbohydrate channeling toward trehalose production rather than glycolysis (Bonini et al., 2000). AM: If this step were up-regulated to a similar degree as other steps (e.g. eight-fold), the glycolytic flux would be twice as strong as in YM, but less than 20% of trehalose, and only 4% of glycogen, would be produced. In addition, earlier simulations have demonstrated that the observed expression pattern of unchanged phosphofructokinase activity is advantageous in terms of NADPH production, as well as the generation of ATP through the glycolytic pathway (Voit and Radivojevitch, 2000).

5.2.2.3. G6P dehydrogenase. Obs: Gene expression for this enzyme increases about six-fold after heat shock. This induction is important for the production of NADPH, which is needed in numerous responses to stresses and toxicity. Even with the six-fold increase, the flux into the pentose phosphate pathway is relatively minor in comparison to the glycolytic flux. YM, AM: Changes in G6P dehydrogenase activity directly and proportionally affect the production of pentoses and NADPH. Because of the small magnitude of this flux (about 10% of the glycolytic flux), changes in the activity of G6P dehydrogenase have little effect on the G6P pool. As a consequence, they affect the trehalose cycle only insignificantly.

5.2.2.4. Phosphoglucomutase. Obs: This enzyme catalyses the first genuine—and readily reversible—step in the production of trehalose and glycogen. Under normal conditions, the reaction clearly favors G6P with an equilibrium constant $K_{eq} = 19$ (Mahler and Cordes, 1969; Reich and Sel'kov, 1981) and a mass action ratio $\Gamma$ at the order of 10 or higher (White et al., 1968, p. 424; Voit, 2000, p. 373)). With most of the mass in the form of G6P, sugar is preferentially channeled into glycolysis, but any excess of G6P is moved toward glycogen storage and thereby prevented from entering glycolysis. The model implementation accounts for the preferred direction of the reaction.

According to the observed expression profile, the phosphoglucomutase genes are up-regulated about 16-fold on average; one of them, PGM2, is actually increased 150-fold, but has few mRNA copies (see Table 1). YM: Analysis of the pathway (Fig. 1) suggests the following advantage of the rapid conversion between G6P and G1P. If plenty of G6P is available, the high activity of phosphoglucomutase allows for rapid storage in the form of glycogen and trehalose. At the same time, if G6P is in demand, stored carbohydrates are to be used. Any usage of glycogen through the glycogen phosphorylase step is effective only if the product of the reaction, G1P, can be channeled rapidly toward increased glycolysis and/or trehalose production (see Fig. 1). This is facilitated by the high activity of phosphoglucomutase.

AM: If the increase in phosphoglucomutase activity does not take place, the model cell generates only two-thirds of the amount of trehalose and a quarter of the glycogen generated by the model wild-type YM. G6P is instead used for a slightly increased glycolytic flux. Increases in phosphoglucomutase activity that are much stronger than observed generate more trehalose and glycogen, but the transcriptional and translational investment yields limiting returns under both, high- and low-glucose conditions. For instance, a three-fold stronger up-regulation (for a total of 48-fold over baseline) yields only 20% more trehalose.

5.2.2.5. UDPG pyrophosphorylase. Obs: Buried in the network of fluxes of the trehalose cycle, this enzyme may not appear to be of particular significance, but in fact it catalyses a crucial, essentially irreversible step (White et al., 1968, p. 425), whose role can be understood from inspection of Fig. 1: Without sufficient conversion of G1P into UDPG, much of the substrate is being returned to G6P through the reversible phosphoglucomutase reaction and subsequently used for glycolysis. In response to heat shock, the UDPG pyrophosphorylase gene is up-regulated 12-fold. AM: Without the increased activity in UDPG pyrophosphorylase, only 3% of the trehalose and 1.5% of the glycogen would be produced in comparison with the wild-type model YM. Furthermore, the internal glucose concentration would decrease to 8%, while G6P would rise. AM: Stronger than observed up-regulation of UDPG pyrophosphorylase yields more glycogen and trehalose, at the cost of slightly reduced glycolysis. The observed expression level may constitute some effective balance.

5.2.2.6. Glycogen synthase. Obs: This step is reversible in principle, but glycogen synthesis is favored under physiological conditions by a factor of about 250 (White et al., 1968). AM: A rather obvious consequence of not up-regulating this step is a reduced concentration of glycogen. In the model without up-regulation, the glycogen level is about 11% of the wild-type model YM. Secondary consequences are huge amounts of UDPG, T6P and trehalose and, subsequently, internal glucose, which rises to 20-fold the normal level under cold conditions. It is interesting how this step, which is rather distant from the glucose pool, actually affects this pool very significantly.
5.2.2.7. Glycogen phosphorylase. Obs: Surprisingly, the gene for this step is much more strongly up-regulated than any other gene in the pathway, with the exception of PGM2. The importance of this step may stem from the fact that it allows the cell to recycle glycogen and use it for trehalose production, glycolysis, and the pentose phosphate pathway. AM: If glycogen phosphorylase activity were kept at baseline, the model cell would produce less than 10% of the amount of glycogen in the wild-type model and generate about 10% more glycolytic flux YM. Additional increases in activity yield limiting returns.

5.2.2.8. Trehalose production. Obs: The genes for the enzymes associated with trehalose production (T6P synthase and phosphatase) are up-regulated 10–20-fold, and T6P synthase activity increases with substrate availability (Lapp et al., 1971; VanderCAMMen et al., 1989). As a consequence, trehalose accumulates swiftly after heat shock, while the T6P concentration remains essentially the same (Blázquez et al., 1993). The latter implies simultaneous and equivalent changes in influx and efflux and is consistent with observations by several authors suggesting that TPS1 and TPS2 form a complex and are co-regulated (e.g. Winderickx et al., 1996; Parrou et al., 1997). It is noted, however, that TPS2 contains four stress elements that respond directly to heat shock, which may ultimately lead to a higher activity in T6P phosphatase than in T6P synthase. YM AM: The amount of trehalose produced correlates directly with the magnitude of up-regulation of the trehalose production complex. Strict one-to-one co-regulation leads to a noticeable increase in T6P, which however is still much smaller than the increase in trehalose. If T6P phosphatase is activated somewhat more strongly than T6P synthase, the T6P concentration remains constant, while trehalose still increases dramatically.

5.2.2.9. Trehalase. Obs: The genes for neutral and acid trehalase are both induced upon heat shock. YM: The level of trehalase activity specified in the model leads to a reasonable level of trehalose accumulation. AM: Significant deviations from the observed level of activation lead either to accumulation or depletion of trehalose. The up-regulation of trehalase is analysed below in detail.

5.3. “Futility” of trehalase activation

The induction of trehalase following heat shock is such a puzzling feature of the trehalose cycle that one might be tempted to question whether it really occurs. Inspecting the diagram of the trehalose cycle (Fig. 1), one could surmise that it would be much more effective for the cell simply to up-regulate the trehalose produc-

...
When the temperature starts to rise, the cell has obviously no information about how long and severe the heat stress might be. It must therefore be able both to produce and degrade trehalose quickly. The mechanisms for accomplishing these tasks fall into two classes. One is transcriptional and the other is based on the degradation, deactivation or repression of the enzymes involved in the trehalose cycle. Observations of Neves and François (1992) indicate that it takes between 10 and 20 min after heat shock, before the activity of the enzymes involved in the trehalose cycle begins to increase. Switching on the corresponding genes, for instance through sphingolipid-based induction (Dickson et al., 1997; Jenkins et al., 1997), occurs very quickly after heat shock, so that the 10–20-min delay is primarily to be attributed to transcription and translation. In comparison to the de novo generation of enzymes through gene regulation, deactivation of enzymes through proteases or through mechanisms like (de-)phosphorylation, (de-)methylation, (de-)glycosylation, or other protein modifications is an order of magnitude faster, even if it involves the action of signal transduction pathways.

The different time-scales of production and deactivation have important dynamic consequences. Suppose, with only slight simplification, that the genes of the TPS complex are switched on within a few minutes of heat shock, remain switched on for a period of about 15 min, and then are turned off. The corresponding enzymes then exhibit an activity profile that stays at baseline for about 10 min (the time needed for transcription and translation) and subsequently increases steadily as long as mRNA molecules are being produced and remain available. This latter period consists of the time interval during which genes are switched on plus the lifetime of the mRNA molecules. Experimental data (De Virgilio et al., 1992; Neves and François, 1992) exhibit this pattern: a period of 10–20 min where enzyme activity is constant, followed by a nearly linear increase that continues for more than an hour (see also Ertugay and Hamamci, 1997). An important consequence of any transcription-based, continued enzyme production is that it cannot easily be stopped at the genome level, even if transcription is blocked. In the case of TPS1/2, this becomes detrimental as soon as trehalose is no longer needed and instead prevents successful recovery from heat stress. Specifically, if the temperature increases only briefly and then returns to normal, the continued and only slowly controllable production of trehalose becomes a liability.

Now consider the response pattern if both trehalose production and degradation are induced at the transcription level, though at a different rate. If the TPS complex is induced at a higher rate than NTH1 and ATH1, the net effect is still an accumulation of trehalose, as required for the immediate response to heat shock. This net accumulation is more expensive in terms of transcription and translation than a sole induction of TPS would be, but it has two dynamic advantages. First, and maybe surprisingly, the rise in trehalose is faster, as can be shown with the following comparison, which is based on the canonical model. Suppose the required level of trehalose in response to heat shock is fixed. To reach this level, the TPS complex is up-regulated 12–16-fold and NTH/ATH six-fold (Stanford Database, 2003), and it takes the model cell a little over 3 min to attain 95% of the desired level. By contrast, if the trehalase genes NTH1 and ATH1 are left un-induced at the baseline level, the TPS genes need to be up-regulated only 15% as strongly to reach the same trehalose level, but it takes 5 times as long (15 min) to reach the 95% level of trehalose accumulation. These times need to be added to the transcription and translation time, which in the latter case would result in a total of about half an hour before the desired trehalose level is reached. Under heat-shock conditions, the speed of reaching the required trehalose concentration is probably critical and may outweigh the additional cost of up-regulating the trehalase genes.

The second dynamic advantage is increased flexibility in control. If heat stress ceases, deactivation of existing enzymes, for instance for trehalose production, is a very fast process. However, it is only effective in reducing the current trehalose concentration if trehalase is available and active. Otherwise, the situation resembles taking the foot off the gas pedal without having the possibility of braking. Production would slow down or come to a halt, but the trehalase concentration would not, or only slowly, decrease. This has been observed in experiments involving NTH1/2 mutants that showed a very slow decrease in trehalose concentration, which was caused exclusively by dilution due to cell population growth (De Virgilio et al., 1994). By contrast, if the cell has both production and degradation of trehalose at its disposal, slight changes in their activities permit precise and almost immediate control. In the model, this is easily demonstrated in the following fashion. The system is moved to its heat-shock steady state where all metabolite concentrations are stable. Now suppose that heat stress ceases and trehalase needs to be removed as fast as possible. If trehalase activity is increased six-fold as observed (Stanford Database, 2003), the trehalose concentration returns to its normal level almost immediately after production stops. By contrast, if trehalase is not induced after heat shock, it takes about 10 min before trehalose returns to normal, even if production is stopped instantaneously.

It may be worth adding that transcription of NTH1 is not induced at temperatures over 40°C (Iwahashi et al., 2000). One can only speculate why this might be the case. First, the cell may need all the trehalose it can produce to prevent proteins from denaturing. Second,
one might hypothesize that at high temperatures the cell has enough time to induce trehalase once the temperature starts dipping below 40°C. In a natural setting, the return from over 40°C to the normal physiological range below 35°C can be expected to last at least several minutes, which might leave sufficient time for activating the trehalase system and ensuring that trehalose is removed for recovery. It is also noted that cell viability is significantly lower at 40°C than at 36°C or 38°C (Ertugay and Hamamec, 1997).

5.4. Initiation of the heat-shock response

An intriguing question is how the trehalose cycle gets started in response to heat shock. A comprehensive answer cannot yet be given, but numerous bits of information are available in the literature. Some of them are restated here and grouped into functional steps, as they relate to the trehalose cycle.

Upon a sufficient increase in temperature, several activities ensue. Very quickly, between 50 and 80 proteins are transiently induced (Davidson et al., 1996; Boy-Marcotte et al., 1999). About 20 of them are heat-shock proteins, whose general role seems to be a mediation of protein–protein interactions or the transport of proteins through membranes. The responses of some of the heat-shock proteins are quite strong (Estruch, 2000). For instance, hsp90 is induced 10–15-fold. However, only hsp104 is directly associated with the immediate heat-shock response and, in particular, with acquiring thermo-tolerance and accumulating trehalose (Sanchez et al., 1992; Davidson and Schiestl, 1996; Schmitt and McEntee, 1996; Estruch and Carlson, 1993; Kobayashi and McEntee, 1993; Vuorio et al., 1993; Varela et al., 1995; Schmitt and McEntee, 1996; Estruch, 2000). STREs of this type have been found in the promoter regions of several genes associated with the trehalose cycle. For instance, it has been established that msn2 and msn4 induce PGMI, GLK1, UGP1, TPS1, and TPS2, as well as the trehalase gene NTH1. Notably, TPS2 contains four—and NTH1/2 three—STRE regions that react with msn2/4 (Gounalaki and Thireos, 1994; Nwaka et al., 1995b; Zähringer et al., 1997, 2000; Wera et al., 1999). Kobayashi and McEntee (1993) demonstrated that multiple STREs act synergistically, and Gounalaki and Thireos (1994) showed specifically that the deletion of two STREs from the promoter region of TPS2 leads to reduced transcription under stress. Zähringer and co-workers (2000) determined that the STREs in the promoter region of NTH1 exert control in a hierarchical fashion and that their physical proximity is important. While the induction of numerous genes is mediated by the interaction of msn2/4 transcriptional factors and STREs, the relative specificity of the transcriptional response of trehalose-associated genes probably derives from “other undefined cis- or trans-acting elements” (Parrou et al., 1997).

The third response of yeast to heat shock is a dramatic activation of its sphingolipid signaling pathway. Recent years have seen an enormous interest in sphingolipids, such as ceramide, with thousands of articles appearing in the scientific literature (Hannun et al., 2001). Sphingolipids have been implicated in a variety of crucial decisions at the cellular level, including differentiation, apoptosis, and the response to various stresses including heat shock (Hannun and Luberto, 2000). Within a few minutes of heat shock, key enzymes of de novo sphingolipid synthesis exhibit increased activity, which may result from temperature-induced changes in their kinetic properties and/or changes in their amount and structure (Wells et al., 1998). The initial enzyme of the pathway, serine palmitoyl transferase is activated in
response to heat (Hannun et al., 2001) and within a few minutes, sphingoid intermediates such as sphinganine and phytosphingosine and their phosphates increase several fold in quantity. Ceramide levels increase more slowly, but remain elevated for two or more hours.

The role of sphingolipids in the response to heat shock could be two-fold (Jenkins et al., 1997). First, sphingolipids are important components of plasma membranes, and since the heat-shock response requires considerable movements of metabolites and mobilization of proteins, the additional quantities of sphingolipids could be crucial. Secondly, sphingolipids have an important regulatory role as second messengers of signal transduction. Dickson and co-workers (1997) showed convincingly that sphingolipid metabolites activate the transcription of TPS2. They suggested that the sphinganine-induced TPS2 expression is mediated by STREs and showed that the msn2 protein is needed for this expression. Another transcription factor, yap1, seems to be involved in heat induction of TPS2 transcription and trehalose accumulation (Gounalaki and Thireos, 1994), but it is not known whether yap1 regulates TPS2 transcription during heat-shock response. yap1 appears to be important in the response to oxidative stress and thus may indirectly have an effect on heat-shock response, through mechanisms of “cross-protection” against various stresses (Estruch, 2000). The Stanford database (Stanford Database, 2003) does not identify YAP1 as up-regulated.

Two crucial steps for triggering heat-shock response in yeast thus appear to be the initiation of sphingolipid signaling and the relocation of msn2 and msn4 from the cytosol to the nucleus. It is unclear whether these two events are initially coincident or whether the heat-altered sphingolipid profile contributes to the mobilization of the “msn2/4 regulon” (Boy-Marcotte et al., 1999). It has been shown, though, that there are msn2/4 independent mechanisms for heat control of trehalose metabolism (Estruch, 2000; Zähringer et al., 2000). The prime target of the interaction between sphingolipids and the msn2/4 regulon is TPS2 with its four STREs. It is not yet fully understood how this cooperation is accomplished, but it is quite remarkable that TPS2 is the point of entry to the trehalose cycle. Not only does the corresponding enzyme activation lead to an immediate increase in the desired trehalose, in the process it depletes T6P, which as a consequence has a weakened inhibition of hexokinase II. The less inhibited and thus more active hexokinase phosphorylates more glucose to G6P, thereby providing more substrate for further trehalose production and diminished repression of T6P synthase by glucose. These chains of events suggest that activation of TPS2 is possibly the optimal step for initiating the trehalose cycle. Supporting the discussion above, it is interesting that the trehalase gene NTH1 contains three STREs in its promoter region (Zähringer et al., 2000).

As is to be expected from a general, strategic point of view, there are checks and balances: If heat shock directly or indirectly induces or activates several steps of the trehalose cycle, one should expect mechanisms to slow down or block these same steps. The cell accomplishes this with the Ras-cAMP–PKA pathway, which in unstressed cells maintains transcription of trehalose-associated enzymes and trehalose itself at low levels and can activate trehalase (Thevelein, 1984a,b; Thevelein and Hohmann, 1995; Dickson et al., 1997; Estruch, 2000). According to Görner and collaborators (Görner et al., 1998, 1999), this signaling pathway counters the effects of heat and constitutes a paradigm for the action of antagonistic pressures and a major control mechanism in signal transduction. The authors suggested that PKA prevents the nuclear accumulation of msn2/4 and thereby sets a threshold for how much signal is required to mount the stress response. PKA may even induce the export of msn2/4 from the nucleus, a process that is modulated by msn5 (Estruch, 2000). Supporting this action of antagonistic processes, Varela and co-workers (1995) found that cAMP/PKA blocks STREs and causes movement of msn2/4 from the nucleus back to the cytosol, an action exactly opposite to the one induced by heat. Most, if not all, genes that are induced by msn2/4 after heat shock are repressed by an excess of cAMP (Boy-Marcotte et al., 1999), and it has been shown that cAMP activates cytosolic trehalase (Thevelein 1984a,b; Thevelein and de Winde, 1999). Thus, any heat-shock response must overcome or control the intracellular cAMP levels. Interestingly, through its inhibitory effect, T6P controls the catalytic activity of hexokinase II, which, via fructose 1,6-bisphosphate, affects the activation of the cAMP-dependent signaling pathway (Thevelein and Hohmann, 1995). Furthermore, neutral trehalase is a substrate of PKA (App and Holzer, 1989; Estruch, 2000), which implies that the activation of NTH1 serves two related purposes, namely the degradation of trehalose after the initial heat-shock response and the induction of the PKA pathway. Of course, heat-shock and the cell’s responses on one hand, and the activation of the cAMP–PKA pathway on the other change over time. Thus, they constitute a dynamic regulatory system that controls the balance between the demands of thermo-resistance and PKA-induced down-regulation (Görner et al., 1999).

6. Conclusion

Mathematical modeling can be applied to biological phenomena in different ways. Its foremost goal is often seen as making predictions of responses of the modeled
entity to untested situations. The analyses shown here have another focus, namely to derive mechanistic and operational explanations for observed regulatory structures and coordinated responses. Casual arguing in terms of causes and effects is often insufficient, even if a system is as small as the trehalose cycle. Similarly, the development and analysis of a single model often do not shed light on the rationale of a particular metabolic feature, may it be in the form of a control signal or the induction of a gene under specific environmental conditions. The approach of choice for such questions is a comparative analysis of two alternative models that differ exclusively in the feature under investigation. Comparisons of this nature are greatly facilitated by a well-structured modeling framework, as it is provided by BST. In BST, every parameter has a unique role and meaning. Each kinetic order $g_{ij}$ or $h_{ij}$ codes directly and quantitatively for the effect that variable $X_i$ has on the production or degradation of $X_j$, respectively. The rate constants $\xi$ and $\beta$ quantify the rates of these processes, and there are no other parameters. Comparisons between alternative structures are therefore objective and precisely focused.

The trehalose cycle consists of a small number of metabolites, which affect each other through a surprising number of control signals. The comparative canonical analysis suggests that each of these is beneficial to the overall functioning of the cycle. Taken together, the signals aid the cell in a complex decision on responses to different external conditions. Under low-glucose conditions, the overriding task for the cell is glycolysis, accompanied by modest channeling towards the pentose cycle, glycogen and trehalose. If plenty of glucose is available in the medium, the cell has the opportunity to store carbohydrates, and because of energetic considerations, the preferred storage compartment is glycogen. Heat-shock conditions mandate yet another fate for some of the glucose taken up from the medium. Trehalose needs to be produced fast, and this production is in direct competition with storage in the form of glycogen.

The key control point for such coordinated channeling is G6P, where the structural and regulatory design of the pathway allows for enough flexibility to respond to different types of demand. Within the confines of this inbuilt structure, specific operational strategies of gene expression regulate the flux distribution at the G6P branch point. This operational control is not static but dynamically responds to the ambient conditions in a highly effective fashion.

The intricacies of the dynamic interactions between opposing signals governing the trehalose cycle remain to be explored in detail. Much of this exploration will require further biochemical and genomic experimentation, but it was shown here that a canonical model can add genuine value to experimental results. The model successfully merged diverse pieces of information from biochemistry, physiology, and genomics, and yielded an integrative structure that provided mechanistic explanations for regulatory and operational features. Beyond these static features, the differential equations permitted assessment of the dynamical advantages of the counter-intuitive induction of trehalase in response to heat shock. The same type of model could be employed to investigate the role of the cAMP-PKA pathway. Furthermore, it is conceptually only a small step to expand the model to account for time-trends in the regulation of genomic expression profiles and for ramifications of the lifetimes of mRNAs and enzymes. These extensions could be the next steps toward a deeper comprehension of the dynamic control of trehalose-associated stress responses that cooperate at the genomic and biochemical levels.

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