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Conditions With High Intracellular Glucose Inhibit Sensing Through Glucose Sensor Snf3 in *Saccharomyces cerevisiae*

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ABSTRACT

Gene expression in micro-organisms is regulated according to extracellular conditions and nutrient concentrations. In *Saccharomyces cerevisiae*, non-transporting sensors with high sequence similarity to transporters, that is, transporter-like sensors, have been identified for sugars as well as for amino acids. An alternating-access model of the function of transporter-like sensors has been previously suggested based on amino acid sensing, where intracellular ligand inhibits binding of extracellular ligand. Here we studied the effect of intracellular glucose on sensing of extracellular glucose through the transporter-like sensor Snf3 in yeast. Sensing through Snf3 was determined by measuring degradation of Mth1 protein. High intracellular glucose concentrations were achieved by using yeast strains lacking monohexose transporters which were grown on maltose. The apparent affinity of extracellular glucose to Snf3 was measured for cells grown in non-fermentative medium or on maltose. The apparent affinity for glucose was lowest when the intracellular glucose concentration was high. The results conform to an alternating-access model for transporter-like sensors. J. Cell. Biochem. 110: 920–925, 2010.

KEY WORDS: GLUCOSE SENSING; TRANSPORTER-LIKE SENSOR; YEAST; MALTOSE; REGULATION

Detection of extracellular nutrients through sensors located in the cell periphery is a widespread phenomenon in living organisms, including the yeast *Saccharomyces cerevisiae*, as reviewed by Holsbeeks et al. [2004]. Sensors of extracellular nutrients in yeast can be divided into two groups: (i) transporter-like sensors, such as glucose sensors Snf3 and Rgt2 [Özcan et al., 1996] as well as the amino acid sensor Ssy1 [Didion et al., 1998; Jørgensen et al., 1998; Iraqui et al., 1999; Klasson et al., 1999], and (ii) the G-protein-coupled glucose sensor Gpr1 [Kraakman et al., 1999]. The three mentioned transporter-like sensors are homologous to corresponding nutrient transporters, but they have not been found to transport significant amounts of the substrates. Some sensors, such as Gap1 [Donaton et al., 2003], Pho84 [Giots et al., 2003; Popova et al., 2010] and Mep2 [van Nuland et al., 2006], are also *bona fide* transporters. Transporting amino acid sensors appear to be important also in mammals, as recently reviewed by Hundal and Taylor [2009].

In this study we investigate sensing through the transporter-like glucose sensor Snf3. The major known role of Snf3 is in regulation of genes encoding glucose transporters [Bisson et al., 1987; Özcan and Johnston, 1995], whereas the range of roles of the similar sensor Rgt2 [Özcan et al., 1996] also comprises regulation of maltose transporter stability [Jiang et al., 1997]. In response to activation of the Snf3 signaling pathway, the co-repressors Mth1 and Std1 are inactivated [Schmidt et al., 1999; Flick et al., 2003]. Removal of Mth1 by ubiquitylation directed by Grr1 and degradation [Spielewoy et al., 2004] results in phosphorylation of repressor Rgt1, and the consequent release of Rgt1 from the promoters of various hexose transporter (*Hxt*) genes leads to increased synthesis of the transporters [Mosley et al., 2003; Kim and Johnston, 2006]. This generates a control mechanism where increase in the extracellular glucose concentration induces expression of more glucose transporters when needed. Some transporters are also glucose repressed at high glucose concentrations. Control of the cytosolic glucose concentration in yeast has perspectives as a model eukaryotic system as well as due to the industrial importance of control of glycolytic flux in yeast.

A model has been suggested for the mode of function of transporter-like sensors based on studies of the amino acid sensor Ssy1 [Gaber et al., 2003; Wu et al., 2006; Poulsen et al., 2008]. Wu et al. [2006] proposed an equilibrium between conformations that are outward facing and inward facing like, for example, the

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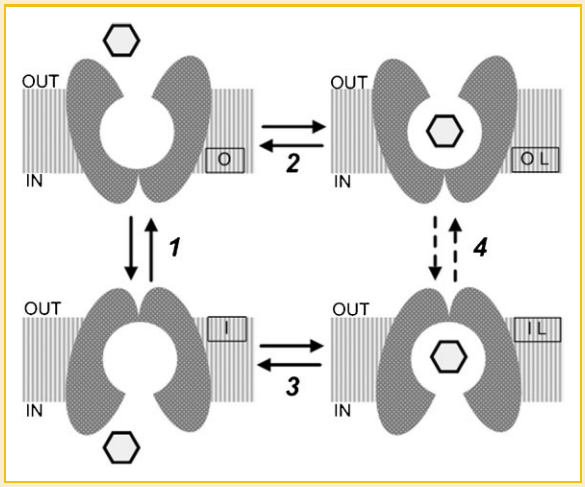

Fig. 1. Model for transporter-like sensors [modified from Wu et al., 2006]. States and reactions corresponding to those of a canonical transporter are presented. The hexagonal object symbolizes the ligand (i.e., glucose or fructose in the current work), and the vertical shaded bars indicate the membrane lipids. The dotted arrows for reaction 4 indicate that for a non-transporting sensor working as proposed for Ssy1p [Wu et al., 2006] or Snf3 (present work), states OL and IL cannot be directly turned into one another. For a real transporter, on the other hand, reaction 4 must be efficient. The outward facing conformation of the sensor (states O and OL) is hypothesized to be signaling, whereas states IL and I are non-signaling. O, outward facing, OL, outward facing, ligand bound; IL, inward facing, ligand bound; I, inward facing. The possibility that occluded states are signaling, as suggested for a transporting sensor [van Zeebroeck et al., 2009], is accommodated by extension of the depicted model by equilibria between outward facing states and occluded states.

structurally similar *Escherichia coli* lactose transporter [Kaback et al., 2007], exposing a centrally positioned ligand binding site to the cell exterior and the cytoplasm, respectively. It was further proposed that the sensor can only shift conformation when ligand is not bound, like in the case of a symporter missing one of its ligands. Finally, it was proposed that the outward facing conformation is the signaling one (Fig. 1). Wu et al. [2006] tested and confirmed the prediction that intracellular ligand competitively inhibits signaling by extracellular ligand in the case of the sensor Ssy1. In other words, the efficiency of nutrient sensing at suitable ranges of concentrations depends on the ratio between intra- and extracellular ligand concentrations. A slightly different model was suggested recently for the case of the amino acid transceptor Gap1, where an occluded, intermediate, conformation was added to the model and proposed to be the signaling one [van Zeebroeck et al., 2009]. Here we investigate how signaling through the sugar sensor Snf3 behaves in relation to intracellular glucose.

MATERIALS AND METHODS

MEDIA

Yeast was grown in synthetic medium (0.67% YNB without amino acids, Beckton & Dickinson, USA) buffered with succinic acid (1%) adjusted to pH 5.5 with NaOH and supplemented with 20 mg/L histidine, 150 mg/L leucine and 20 mg/L tryptophan. As carbon source was used a combination of 5% glycerol, 2% ethanol, 1% glutamic acid, and 1% aspartic acid (non-fermentable medium), or 2% maltose. Cultures were grown in baffled shake flasks with culture volume less than 10% of the flask volume.

STRAIN CONSTRUCTION

The yeast strains used in this study are shown in Table I. Parental yeast was the glucose transport-deficient strain EBY.VW1000 (*Δhxt1* through *Δhxt17*, *Δgal2*) [Wieczorke et al., 1999]. We integrated in *MTH1* as a C-terminal fusion a sequence encoding two copies of the Z domain of *Staphylococcus aureus* protein A to be used as an epitope tag (ZZ-tag), analogously to Poulsen et al. [2005]. The Z domain sequence was integrated immediately at the end of the coding sequence of *MTH1* using *URA3* as a marker. *RGT2* was furthermore deleted by substitution with *LEU2* to yield strain M5965. To construct strain M5967, *HXK2* was deleted by substitution with *TRP1* in the M5965 strain background. To construct strain M5966 lacking both Snf3 and Rgt2, *RGT2* was deleted by substitution with *LEU2* in strain EBY.VW2000 (*Δhxt1* through *Δhxt17*, *Δgal2 Δsnf3*) [Wieczorke et al., 1999]. For strain M5897, Snf3 was tagged with the Z domain epitope tag analogously to the case of Mth1. Standard molecular biology methods were used for all constructs.

MEASUREMENT OF MTH1 DEGRADATION

Protein quantification protocol was adapted from Poulsen et al. [2005]. Cultures were inoculated at an optical density (OD) of 0.05, and the experiments were performed when OD reached 0.5–1 (mid-exponential phase). Cells from a single batch of culture were exposed to a series of inducing sugar concentrations in a volume of 2.5 ml for exactly 10 min, after which proteins were instantly extracted by alkaline lysis [Brandt, 1991] with 20 mM DTT as reducing agent. Protein extraction was performed for 1 ml of culture-glucose mixture, and all protein from this solution was precipitated with trichloroacetic acid and dissolved in 25 μl of Western blot loading buffer [Poulsen et al., 2005]. Western analysis was performed as previously described using the Storm fluorescent scanner (Molecular Biosystems) [Poulsen et al., 2005]. The Mth1 levels in the sample series were normalized against a sample without inducing sugar within the same gel. Degradation of the Mth1 protein was quantified by image analysis of the Western blot using ImageQuant software (Molecular Biosystems). After quantification of tagged protein, the blots were stained with Ponceau S (Sigma–Aldrich) to verify that equal amounts of total protein were loaded in the wells. Experiments were made in at least duplicate.

Mth1 levels were normalized for the control sample and plotted against sugar concentration in a semi-log plot. Data were fitted to the equation $y = y_0 + (ax^b)/(c^b + x^b)$ using SigmaPlot 2000 (SPSS Inc., IL) and the constraints: $y_0 > 0$ and $b = -1$. The median effective concentration (EC_{50}), is equal to the constant c. Small leakage of glucose (0.5–1 mM) was determined and taken into account in the EC_{50} determination for glucose. Thus glucose concentration was measured in culture filtrate, and the amount of leaked glucose was added to the amount of externally added glucose to obtain the total glucose concentration of the test samples. Standard error of the mean (SEM) of EC_{50} values was less than 24%.

TABLE I. Relevant *S. cerevisiae* Strains

Strain	Genotype	References
CEN.PK2-1C	*MATa leu2-3 ura3-52 trp1-289 his3-Δ1 MAL2-8^c SUC2*	Entian and Kötter [1998]
EBY.VW1000	CEN.PK2-1C *Δhxt1 through 17, Δgal2*	Wieczorke et al. [1999]
EBY.VW2000	CEN.PK2-1C *Δhxt1 through 17, Δgal2 Δsnf3*	Wieczorke et al. [1999]
M5965	EBY.VW1000 *MTH::MTH-ZZ rgt2::LEU2*	This work
M5966	EBY.VW2000 *MTH::MTH-ZZ rgt2::LEU2*	This work
M5967	EBY.VW1000 *MTH::MTH-ZZ rgt2::LEU2 hxk2::TRP1*	This work
M5987	EBY.VW1000 *SNF3::SNF3ZZ*	This work

MEASUREMENT OF INTRACELLULAR GLUCOSE CONCENTRATIONS

Intracellular glucose was determined after quenching in cold methanol according to Loret et al. [2007], in duplicate. Glucose was extracted from pelleted cells with boiling ethanol [Loret et al., 2007]. A commercial glucose assay (GAGO20, Sigma–Aldrich) was used according to manufacturer's recommendations except that glucose levels were determined from reaction rates with relevant controls. Cell volume was taken to be 2 ml per g dry weight [Guijarro and Lagunas, 1984]. The relation between cell dry weight and optical density was 0.25 mg cells per OD unit for all conditions except 0.18 mg cells per OD unit for the *hxt*-null *Δhxk2* cells grown on glycerol. SEM of the intracellular glucose concentration was less than 10%.

RESULTS AND DISCUSSION

Sensor for extracellular glucose Snf3 [Bisson et al., 1987; Özcan et al., 1996] is an integral plasma membrane protein that shares high sequence similarity with glucose transporters but does not transport glucose. It was previously found that cytosolic glucose has a negative influence on glucose uptake by a so far unknown mechanism [Teusink et al., 1998]. Here we assessed signaling through Snf3 in varying intracellular glucose concentrations, in the perspective of the previously suggested model for function of transporter-like sensors [Wu et al., 2006]. To separate extra- and intracellular sugar pools, we used yeast mutants lacking mono-hexose transporters Hxt1 through Hxt17 and Gal2 [Wieczorke et al., 1999]. The *RGT2* gene, encoding a similar glucose sensor with lower affinity [Özcan et al., 1996], was deleted in all strains to simplify interpretations. Upon exposure to glucose, a signal from Snf3 causes degradation of co-repressor Mth1, whereby repression of major hexose transporter genes is relieved. We quantified signaling through Snf3 by measuring degradation of epitope-tagged Mth1 protein after addition of glucose at various concentrations. Degradation of Mth1 was chosen as a means of detection rather than *HXT* transporter gene expression as the Mth1 degradation is the first measurable step in the glucose signaling cascade, which reduces the risks for side-effects that may emerge if a step further down in the pathway is measured. The cells were grown in a non-fermentable or maltose mineral medium, after which samples of the cell culture were incubated with glucose. The amount of Mth1 protein was determined by Western blotting. In initial kinetic experiments (Fig. 2) the Mth1 level came down to a plateau after 10 min of incubation. When cycloheximide (100 μg/ml) was added together with the glucose, the Mth1 level went to zero, which indicates that there were concomitant degradation and synthesis of Mth1 when cycloheximide was not added. In all experiments reported below, we incubated the cells with glucose for 10 min without addition of cycloheximide. This time was considered sufficiently short to reduce any side-effect of glucose that might arise through transcriptional regulation.

The Mth1 levels measured after incubation in various glucose concentrations were plotted as a function of extracellular glucose (Fig. 3). The plots were fitted to the relation expected if signaling is linear with the number of Snf3 molecules occupied with glucose in an externally accessible single binding site, and therefore hyperbolic toward concentration (sigmoid in a semilog plot). The concentration at which signaling is half of its maximum (EC_{50}) was regarded as an apparent dissociation constant of extracellular glucose to Snf3. We found an EC_{50} of extracellular glucose of 0.44 mM toward cells grown in non-fermentable medium (Fig. 3). When Snf3 was deleted (Strain M5966), no degradation of Mth1 was observed in response to glucose.

To manipulate intracellular glucose concentration, we varied the carbon source. Glucose transport-deficient cells grown on maltose are known to accumulate glucose that is formed by intracellular cleavage of maltose [Jansen et al., 2002]. We found an intracellular glucose concentration of 3.3 mM in cells grown in non-fermentable medium, whereas the intracellular glucose concentration was 18 mM in maltose-grown cells. These concentrations are in line with previously published data [Jansen et al., 2002]. The EC_{50} of extracellular glucose was 1.7 mM for cells grown in maltose-containing medium, which is an increase of about fourfold (Fig. 3)

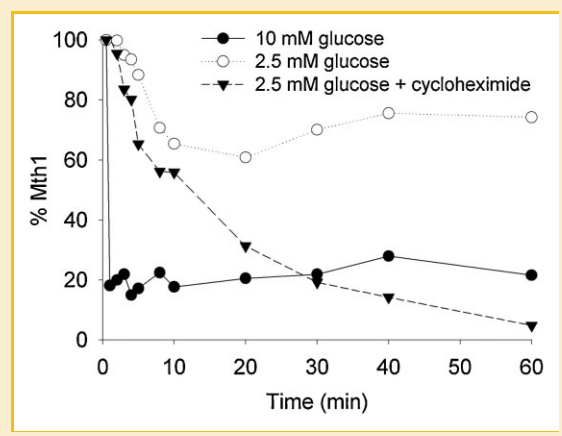

Fig. 2. Degradation of Mth 1 in response to variable amounts of sugar with and without cycloheximide.

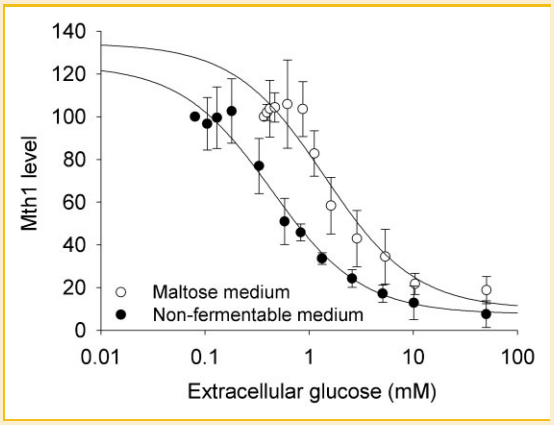

Fig. 3. Glucose sensing by Snf3 measured by Mth1 degradation (arbitrary units) for strain M5965 grown in non-fermentable medium or in maltose medium.

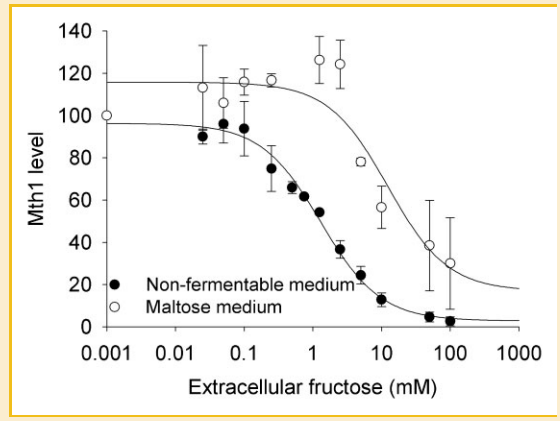

Fig. 4. Fructose sensing by Snf3 measured by Mth1 degradation (arbitrary units) for strain M5965 grown in non-fermentable medium or in maltose medium.

compared with cells grown in non-fermentable medium. This suggests that intracellular glucose has an inhibitory effect towards the signaling.

We interpret the lower responsiveness of Snf3 seen when the internal glucose concentration is high to reflect inhibition of Snf3 by intracellular glucose as predicted by our model for transporter-like sensors [Fig. 1, Wu et al., 2006]. This model proposes two conformations of the sensor. However, we wish to point to the possibility that the signaling conformation is one that is in equilibrium with, but different from, the outward facing conformation, for example, an occluded state, where the ligand binding site is neither accessible from the outside nor from the inside. Reasons to consider this possibility are that (i) the existence of an occluded conformation has been suggested in several transporters, for example, $LeuT_{Aa}$ [Yamashita et al., 2005], and (ii) the finding that certain competitive inhibitors of transport through the *transporting* sensors Gap1 and Pho84 do not trigger signaling, that is, they presumably are not able to trigger a conformational change [van Zeebroeck et al., 2009; Popova et al., 2010], arguing that the signaling conformations of Gap1 and Pho84 are occluded. Our data for Snf3 do not distinguish this possibility from that of the simpler model depicted in Figure 1, and we do not find either possibility more likely than the other in the case of non-transporting transporter-like sensors.

The model predicts that signaling through Snf3 by another extracellular elicitor is affected by intracellular glucose in a similar way. We found this to be the case for fructose, which exhibited an EC_{50} of 1 mM toward cells grown in non-fermentable medium and 12 mM toward maltose-grown cells (Fig. 4). These values were derived without taking leaked glucose into account, since this was not straightforward because of the different potencies of glucose and fructose. We take this to be the reason why the ratio between the EC_{50} on maltose and that on non-fermentable medium turned out somewhat different from the case of glucose sensing.

We considered the possibility that a metabolite downstream of glucose phosphorylation, rather than glucose, is responsible for inhibition of signaling. We therefore repeated the experiment with a *hxt*-null strain lacking the major hexokinase, Hxk2, consequently having low capacity for glucose phosphorylation. Although two other enzymes with hexokinase activity, Hxk1 and Glk1, are present in yeast, overall glucose phosphorylating activity is significantly lower in cells with an *hxk2* deletion [Diderich et al., 2001; Schuurmans et al., 2008]. Deletion of *hxk2* did not essentially change responsiveness of the *hxt*-null cells grown in non-fermentable medium, where the intracellular glucose concentration was 18 mM (Fig. 5). The growth rate of the *hxt*-null *Δhxk2* strain on maltose ($0.16\,h^{-1}$) was similar to that of the parental strain ($0.15\,h^{-1}$), indicating that the *hxk2* deletion did not have a dramatic effect on vitality. However, the cells contained 310 mM glucose (Fig. 5), that is, a 17-fold increase, and no signaling in response to extracellular glucose was detected. No significant degradation of Mth1 protein was observed with extracellular glucose concentrations up to 500 mM (Fig. 6.). This finding is consistent with intracellular glucose being the direct inhibitor of Snf3 signaling, and does not fit easily with downstream metabolites being responsible, even if a known role of Hxk2 in glucose repression system is

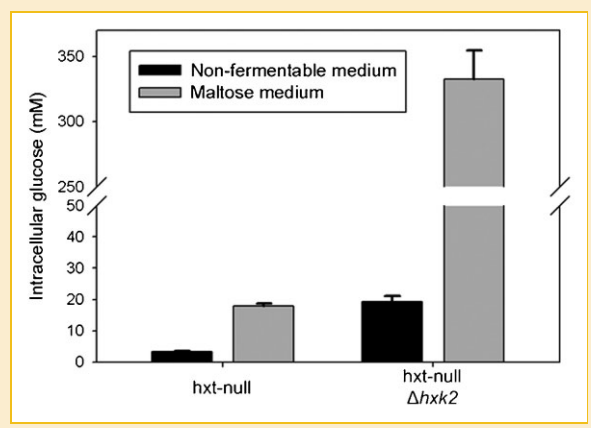

Fig. 5. Intracellular glucose concentrations in WT (M5965) and *Δhxk2* (M5967) cells grown on non-fermentable medium or maltose medium.

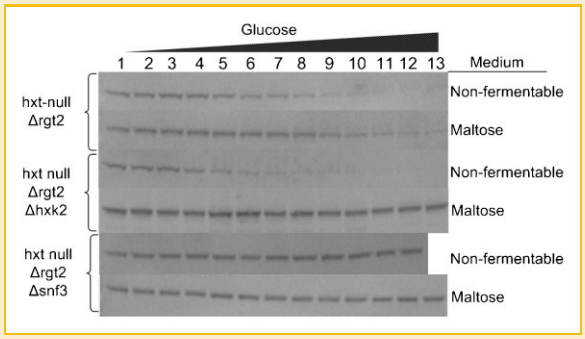

Fig. 6. Western blot of Mth1 degradation in response to glucose. Strains M5965 (Δ*rgt2*), M5967 (Δ*hxk2*) and M5966 (Δ*rgf2*, Δ*snf3*) were grown on non-fermentative medium or maltose medium. Concentration of glucose added in samples 1–13: 0, 0.025, 0.05, 0.1, 0.25, 0.5, 0.75, 1.25, 2.5, 5, 10, 50, and 100 mM glucose, respectively.

considered. It also adds data for correlating EC_{50} with intracellular glucose, although EC_{50} cannot be determined for maltose grown cells as Mth1 is not degraded at all.

Among contributing/alternative mechanisms we also considered the level of Snf3. In separate experiments, *SNF3* was thus tagged as described above (strain M5987). The ZZ-tagged Snf3 protein was present at similar levels, within a factor of 1.1 ± 0.64 under both growth conditions (Fig. 7). The Snf3 protein was detected as two bands, the function of which was not further investigated. Addition of glucose did not change the Snf3 level within 10 min. The amount of Mth1 was a factor of 1.6 higher in cells grown on maltose than on the non-fermentable medium; this might contribute as a mechanism in the unlikely case that the protein degradation system was saturated. Degradation of co-repressor Std1 also makes part of the signal pathway in parallel to degradation of Mth1. Std1 protein was present at same, relatively low, level in both non-fermentable and maltose medium (data not shown), and is not expected to interfere with the analysis.

Snf3 function has been seen as a way of securing major glucose transporters to be made only when needed. The present work predicts a function in intracellular glucose homeostasis; at constant extracellular glucose concentrations within a particular range, Snf3 will work as a sensor of intracellular glucose responding to increasing concentrations by reduced glucose uptake and vice versa. The evidence in this work does not exclude the possibility that the site of effect of the intracellular glucose may not be in Snf3 but elsewhere in the signaling pathway upstream of Mth1 degradation. Control of the intracellular glucose concentration is important in the control of glycolytic flux. We suggest that it is additionally important for limiting glucose toxicity; sugars and even more so some of their metabolites react with amino groups of proteins in processes known as glycation. As reviewed by Suji and Sivakami [2004] this is highly important for aging processes in humans, and Gomes et al. [2005] found that glucose concentration in the growth medium of yeast has a strong influence on the intracellular methyl glyoxal concentration, which in turn relates directly to protein glycation.

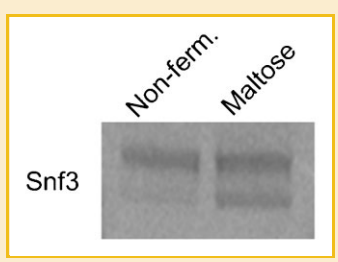

Fig. 7. Western blot of Snf3 protein abundance in non-fermentable medium (left) and in maltose medium (right) (strain M5987).

The present study suggests a generalization of the model of Wu et al. [2006], which rationalizes the effect of intracellular ligand on sensing by transporter-like sensors, and predicts that, in proper ranges, the ratio of extra- and intracellular ligand concentrations is sensed.

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