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A genetically encoded Förster resonance energy transfer sensor for monitoring in vivo trehalose-6-phosphate dynamics

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Abstract

Trehalose-6-phosphate is a pivotal regulator of sugar metabolism, growth, and osmotic equilibrium in bacteria, yeasts, and plants. To directly visualize the intracellular levels of intracellular trehalose-6-phosphate, we developed a series of specific Förster resonance energy transfer (FRET) sensors for in vivo microscopy. We demonstrated real-time monitoring of regulation in the trehalose pathway of *Escherichia coli*. In *Saccharomyces cerevisiae*, we could show that the concentration of free trehalose-6-phosphate during growth on glucose is in a range sufficient for inhibition of hexokinase. These findings support the hypothesis of trehalose-6-phosphate as the effector of a negative feedback system, similar to the inhibition of hexokinase by glucose-6-phosphate in mammalian cells and controlling glycolytic flux.

Keywords

Trehalose-6-phosphate; FRET sensor; Glycolysis regulation

Trehalose, a disaccharide formed by two glucose moieties, is ubiquitously found in bacteria, yeast, plants, insects, and nematodes [1–4]. It is used as a carbon source, serves as an intracellular storage carbohydrate, and also helps organisms to cope with osmotic and thermal stress. The metabolic pathways for de novo biosynthesis of trehalose from hexoses or its catabolism are tightly intertwined. Precise coordination of trehalose production and degradation is crucial to ensure fitness in the face of environmental and nutritional changes. A key component of the trehalose pathway is trehalose-6-phosphate (T6P).² Beyond its role as an immediate neighbor of trehalose in metabolism, T6P acts a signaling molecule to regulate sugar metabolism and physiological processes. For instance, T6P regulates sugar use, growth, and development in plants [5,6].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ab.2014.12.019>.

²Abbreviations used: T6P, trehalose-6-phosphate; FRET, Förster resonance energy transfer; PCR, polymerase chain reaction; eCFP, enhanced cyan fluorescent protein; IPTG, isopropyl β-D-1-thiogalactopyranoside; DTT, dithiothreitol; SCM, synthetic complete medium.

The flexibility and dynamic regulation of trehalose metabolism is nicely exemplified for *Escherichia coli*. During growth on hexoses and other carbon sources, *E. coli* can produce trehalose by treha-lose-6-phosphate synthase (OtsA) and trehalose-6-phosphate phosphatase (OtsB) to convey stress protection [7,8]. If trehalose is present in the environment, it can be taken up and phosphorylated by the phosphotransferase system (TreB) to release T6P into the cytoplasm. Catabolism of T6P to glucose and glucose-6-phosphate is catalyzed by trehalose-6-phosphate hydrolase (TreC). Expression of the TreB/C operon depends on intracellular T6P levels and ensures efficient use of trehalose as a carbon source [9]. In conditions of high osmolarity, however, the expression of the trehalose transporter TreB is repressed [8]. Under such conditions, trehalose is first hydrolyzed by the periplasmic trehalase TreA and then taken up by the cells as glucose.

The regulatory role of T6P can also span beyond the boundaries of the trehalose pathway. In the budding yeast *Saccharomyces cerevisiae*, it was shown that T6P inhibits hexokinases I and II in vitro and, thus, might act as a gatekeeper for the glycolysis influx [10]. This view is consistent with the observation that mutants deficient in T6P synthase (Tps1) are unable to grow on glucose because it lacks T6P to prevent depletion of adenosine triphosphate (ATP) by the hexokinase [3]. However, the in vivo relevance of T6P hexokinase inhibition has been questioned. Because the synthase Tps1 forms a protein complex with the T6P phosphatase Tps2, which produces trehalose from T6P [11,12], it has been speculated that channeling of T6P between these two enzymes would not allow accumulating sufficient free T6P in the cytoplasm to inhibit the hexokinases.

A better understanding of the physiological role of T6P, potentially including hexokinase inhibition, calls for means to measure in vivo T6P dynamics. So far, the quantification of T6P has been limited to whole-cell or whole-tissue extracts and entails laborious chromatographic- or enzymatic-based methods [13–15]. These methods are destructive and fail to selectively measure intracellular free T6P or monitor its transients over time in the same cell. An additional challenge is the low T6P concentrations often found in vivo, particularly in plants. Here, we present a novel genetically encoded biosensor based on Förster resonance energy transfer (FRET) to measure T6P concentration in vivo. FRET sensors consist of a chimeric protein composed of a ligand-binding protein fused to two fluorescent proteins that form the FRET pair. The binding of the target molecule triggers conformational changes on the structure of the sensor construct and changes the amount of energy transferred between the fluorophores. The concentration of the target molecule directly translates into a change in fluorescent emission. We used the repressor TreR from *E. coli* as the scaffold for developing the T6P-TRACK series of sensors with binding affinity constants in the range of physiological T6P concentrations (μM to low mM) [13,16]. The in vivo performance of the sensors was characterized and validated in *E. coli*. We then applied our T6P sensors to address the controversy on the free cytosolic levels of T6P in *S. cerevisiae*.

Materials and methods

Chemicals and strains

Analytical-grade chemicals were purchased from Sigma–Aldrich. Enzymes for DNA cloning were purchased from New England Biolabs. The *E. coli* strains TOP10F⁺ and BL21(DE3) were purchased from Stratagene. W303 MATa (*ura3*, ADE2, TRP1, HIS3, LEU2) yeast strains were used.

Sensor construction and cloning

The gene of TreR (<http://ecocyc.org>, accession No. EG12202) from *E. coli* was amplified by polymerase chain reaction (PCR) using primers containing *SpeI* or *KpnI* restriction sites (see Table S1 in online Supplementary material). The PCR product was digested with *SpeI* and *KpnI* and then ligated into a pGWF1 vector [17] containing the fluorescent protein encoding regions. The resulting plasmid generated the genetic fusion of the fluorescent proteins eCFP (enhanced cyan fluorescent protein) and Venus (yellow fluorescent protein) with the full-length TreR (eCFP–TreR–Venus). pGWF1 is derived from the pRSETb expression vector and places the recombinant fusion gene under control of a T7 promoter. Truncations of TreR were prepared from the full-length construct eCFP–TreR–Venus by PCR amplification using the listed pairs of primers (Table S1). The amplicons were then ligated to pGWF1 using the *SpeI* and *KpnI* sites as described above. For the generation of sensor mutants with reduced affinity for T6P, point mutations were introduced by a QuikChange Kit (Stratagene) using the listed primers (Table S1). For expression in yeast, the sensors were cloned into a pDRf1 plasmid [18]. The sequences of the generated clones were all confirmed by Sanger sequencing (Microsynth, Switzerland).

In vitro characterization of T6P–TRACK family of sensors

E. coli cells BL21(DE3) were transformed with vectors carrying the sensor constructs and cultivated at room temperature in the dark for 2 to 3 days in liquid LB medium supplemented with 100 mg L^{−1} ampicillin. No isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the cultures in order to allow the slow expression and proper folding of the sensors. Cells were then harvested, resuspended in 20 mM sodium phosphate buffer (pH 7.4), 0.5 M NaCl, 20 mM imidazole, and 2.5 mM dithiothreitol (DTT) (washing buffer), and then disrupted by sonication. The clarified cell lysate was then loaded onto a nickel column GraviTrap (GE Healthcare) and washed with washing buffer. The purified sensor was eluted from the column using 20 mM sodium phosphate buffer (pH 7.4), 0.5 M NaCl, 500 mM imidazole, and 2.5 mM DTT. The buffer of the samples was then finally exchanged to 20 mM Mops (pH 7.4), 160 mM NaCl, 20 mM MgCl, and 5 mM DTT (assay buffer) by ultrafiltration. In vitro characterization of the sensors was done in the assay buffer at room temperature using samples not older than 1 day and at a protein concentration of 100 to 500 nM. Fluorescence emission spectra and eCFP and Venus intensity measurements were performed on a TECAN Infinite 200 plate reader (TECAN, Austria) in 96-well plates. Ratiometric FRET measurements were done by excitation at 430 nm (bandwidth 9 nm) and reading emission at 480 nm (eCFP) and 530 nm (Venus) (bandwidth 20 nm). Dissociation constants (K_d) and Hill coefficients (n) were determined by fitting the titration data to the Hill equation [19] using nonlinear least-squares fitting in Microsoft Excel [20]:

$$\frac{(R - R_0)}{(R_{max} - R_0)} = \frac{[T6P]^n}{K_d^n + [T6P]^n}, \quad (1)$$

where R is the Venus/eCFP ratio, R_0 represents the Venus/eCFP ratio at 0 mM T6P, R_{max} is the maximum ratio observed, and $[T6P]$ is the ligand concentration.

In vivo T6P measurements in *E. coli*

BL21(DE3) cells expressing the constructs T6P-TRACK-20 and T6P-TRACK-control were cultivated overnight in 50 ml of LB medium with 100 mg L⁻¹ ampicillin and without IPTG in 500-ml Erlenmeyer flasks with orbital agitation (120 rpm) in the dark at room temperature without controlled atmosphere. The cells were then harvested by centrifugation and resuspended in M9 minimal medium without any carbon source to an OD₆₀₀ around 1.8 to 2.5. To measure the intracellular accumulation of T6P, different aliquots of cells received a pulse of a range of trehalose concentrations after 45 min of carbon starvation. Trehalose stock solutions used in the experiments with *E. coli* were also prepared in M9 medium at concentrations ranging from 1 to 100 mM. Typically, 2 µl of trehalose solution was added to 200 µl of cell culture. Emission ratios were corrected for fluorescence changes not related to FRET by subtracting the Venus/eCFP values obtained with samples expressing T6P-TRACK-control from those expressing T6P-TRACK-20. FRET ratios were recorded approximately 20 to 45 s after trehalose addition. To evaluate the effect of osmotic stress on trehalose uptake, transformed *E. coli* cells were cultivated overnight using similar conditions as described above but in LB medium supplemented with 75 or 250 mM NaCl. The cells of both samples were then resuspended in M9 minimal medium containing the same amount of salts and starved for 45 min prior to the addition of 1 mM trehalose. The concentration of T6P accumulated intracellularly on the addition of the same amount of trehalose was dependent on the age of the culture, cultivation temperature, and starvation time.

In vivo T6P measurements in *S. cerevisiae*

Yeast cells were transformed with pDRf1 plasmids [18] containing either T6P-TRACK-20, T6P-TRACK190, or T6P-TRACK-control using a standard lithium acetate transformation protocol. Several colonies were inoculated into 10 ml of synthetic complete medium lacking uracil (SCM-Ura) [21] with 1% glucose and then cultivated for 24 h at 30 °C in the dark with shaking. For imaging, 200 µl of cells from the 24-h stationary phase cultures was collected by centrifugation and resuspended in 1 ml of SCM-Ura without glucose. After 5 s of sonication, cells were loaded into a fresh chamber of a Y04C imaging plate (ONIX System, CellASIC, Hayward, CA, USA). The flow was set to 8 psi throughout the experiment. Cells were placed for 10 min in SCM without glucose before being subjected to imaging. Cells then received another 10 min of SCM without glucose and a one-step increase to SCM with a range of glucose concentrations as shown in Fig. 6 (see Results). Microscopy was performed using a Zeiss Observer Z1 microscope with an automated stage and a Plan-Apo 63X/1.4NA oil immersion objective. Automatic hardware-based focusing was performed by Definite Focus. The blue fluorescence (480/40 nm) and yellow fluorescence (535/30 nm) on excitation at 436 ± 25 nm, as well as phase images, were recorded every 30 s. Images were analyzed using custom Matlab software [22] that

automatically tracks cells and quantifies fluorescence intensities. Mean Venus/eCFP pixel intensities for each cell were determined from 5 min before until 10 min after the step increase in glucose. To avoid potential artifacts from differential plasmid-based expression, only cells with fluorescent intensities between 15 and 85% of the maximal intensity were used for analysis. All sensor and control cells, typically between 10 and 20, were averaged separately. To obtain the corrected FRET signal, the FRET ratio of the control cells was subtracted from the cells harboring the sensor. A three-point moving average was used to smooth traces.

Results

A FRET-based sensor for T6P

To construct a set of protein-based FRET sensors for monitoring the intracellular T6P levels, we require a scaffold-binding protein that translates ligand concentration into a conformational change and in turn rearranges the flanking fluorescent proteins. To achieve this, we used the trehalose repressor (TreR) from *E. coli* [9] as the starting scaffold. TreR belongs to the LacI family of DNA-binding proteins, which typically regulate genes involved in sugar and nucleotide metabolism [23]. Like most members of its family, TreR is a homodimeric protein composed of a DNA-binding domain and an effector-binding domain. In the absence of T6P, TreR binds to the operator palindromic sequence of the TreB/C operon and prevents its expression. Once T6P is available, it binds to the repressor, triggering structural changes that promote the release of the operator DNA and activation of expression. Although TreR is able to bind both trehalose and T6P in the same site, only the latter acts as an inducer and reduces the affinity of the repressor for the operator region [9]. To construct our sensor, we fused the TreR protein to eCFP and Venus [17]. The product of this fusion exhibited spectroscopic features characteristic of FRET sensors with emission bands centered at 480 and 530 nm that originated from the eCFP and Venus proteins, respectively, on CFP excitation (Fig. 1). In vitro titration of the purified chimeric protein with increasing amounts of T6P caused a change in the Venus/eCFP ratio of approximately 0.20 (~11%; see Fig. S1 in Supplementary material).

For in vivo application, it is important to maximize the dynamic range of changes in the acceptor/donor ratio. Otherwise, small fluorescence changes may become indistinguishable from measurement noise in the more complex cellular context. One way to enhance the dynamic range of a FRET sensor is by building constructs in which the sandwiched ligand-binding protein has been truncated. This may lead to a different spatial arrangement of fluorophores and improve the FRET signal changes [24,25]. We sought to improve the dynamic range of the initial T6P sensor by preparing derivative constructs containing 14 different truncated versions of TreR (Fig. 2). The 60 residues at the N terminus of TreR fold into the DNA-binding domain of the repressor [9], and we tested the effect of partial or total removal of this domain in 9 different constructs. We also designed 5 additional truncated forms of TreR based on the three-dimensional structure of the T6P-binding domain [26]. In these constructs, we removed residues that are not directly involved in ligand binding and that could shorten the distance between the fluorophores or modify their spatial orientation. Deletion of 62 or 40 residues from the N terminus improved the dynamic range. Truncation

of 62 residues enhanced the amplitude of FRET changes but also inverted the sign of Venus/eCFP change caused by T6P addition (Fig. 2). This implies a substantial modification in the kind of structural rearrangement triggered by T6P binding compared with the other constructs (truncation of 60 N-terminal residues had a similar effect). Truncation of 40 N-terminal residues from the DNA-binding domain improved the amplitude of ratiometric change by approximately 50% without modifying its sign (Fig. 2). This improved sensor version was preferred because positive ratiometric changes can be more easily discriminated from photobleaching effects that result in a gradual drop in the acceptor/donor ratio over time [27] due to Venus being more photosensitive than eCFP [28,29]. An additional positive aspect of the complete or partial removal of the TreR DNA-binding domain is that we can rule out the risk of having the construct inadvertently interacting with DNA. The deletion of 40 residues of the DNA-binding domain (as well as at the attachment of the fluorescent proteins) had only a small effect on the affinity of the TreR moiety for T6P. The apparent dissociation constant of the sensor ($K_d = 22 \pm 3 \mu\text{M}^{-1}$) is comparable to affinity of the unmodified TreR ($K_d = 10 \mu\text{M}^{-1}$) [9]. This T6P sensor construct was named T6P-TRACK-20.

Development of control construct and sensors with reduced affinity

Next, we set out to modify T6P-TRACK-20 to obtain a nonbinding variant that can be used in vivo to correct for fluorescence changes that are caused by physiological factors such as pH, ionic strength, and osmolarity rather than ligand binding. Based on the crystal structure of TreR [26], we mutated the T6P binding site using site-directed mutagenesis. To abolish the binding ability of the sensor, we substituted Arg71 for a leucine residue (R71L). The guanidinium group of Arg71 forms a hydrogen bond with the oxygen of the glycosidic bond between the glucose moieties of the T6P and also forms a salt bridge with the negatively charged phosphoryl group of T6P [26]. Consistent with this picture, the R71L mutation led to a construct showing no significant FRET change even at T6P concentrations as high as 10 mM (Fig. 3A and B). The R71L mutated version of the sensor was named T6P-TRACK-control and was used in all in vivo experiments described in this work to correct for fluorescence changes not related to ligand binding.

To cover a higher concentration range, we engineered two additional versions of the sensor with reduced affinity for T6P. In the crystal structure of TreR in complex with T6P [26], the hydroxyl group of the Ser76 side chain interacts via direct or water-mediated hydrogen bonds to the phosphoryl group of the effector molecule. Substitution of Ser76 for an alanine lowered the affinity for T6P and increased the K_d of the sensor to $119 \pm 12 \mu\text{M}^{-1}$. This modified sensor was named T6P-TRACK-120. Finally, mutation of Arg147 to a leucine caused an even stronger decrease in the affinity for T6P. The guanidinium group of Arg147 forms multiple hydrogen bonds to oxygen atoms of the sugar moiety of T6P [26], and its removal increased the affinity constant to $186 \pm 14 \mu\text{M}$. We named this third sensor T6P-TRACK-190. All of the T6P-TRACK sensors have Hill coefficients in the range of 1.4 to 1.6 (Table 1), which indicates positive cooperativity in T6P binding. Given that TreR binds only one T6P molecule per monomer, this result suggests that the sensor preserves the dimeric nature of TreR [9].

Selectivity of T6P sensors

To examine the selectivity of our sensors, we tested the response of T6P-TRACK-20, T6P-TRACK-120, and T6P-TRACK-190 in vitro to compounds structurally related to T6P. We also verified the selectivity against compounds that have lower structural similarity to T6P but are commonly abundant in biological systems such as plants and yeast. The addition of trehalose, glucose-6-phosphate, glucose-1-phosphate, glucose, sucrose-6-phosphate, or phosphate ion at concentrations as high as 10 mM did not trigger any significant FRET changes on any of the sensors (Fig. 3B). Even the mutated versions, where protein-ligand interaction points were disrupted, showed remarkable selectivity for T6P. The small changes in the Venus/eCFP caused by the molecules other than T6P are in the same range as the measurement noise. These results highlight the excellent selectivity of the T6P-TRACK family of sensors and suggest their suitability for in vivo experiments. In addition, we tested the performance of all sensors in the presence of trehalose and confirmed that although the disaccharide had an effect on the affinity of the sensor, presumably due to competition with the target molecule, it did not prevent it from responding to T6P addition (Fig. 4).

The selectivity of our T6P sensors supports the view that only T6P can trigger conformational changes in TreR. Although the crystal structures of TreR with T6P showed nearly identical folding as the TreR-trehalose complex [26], our results are consistent with the previous reports showing that only T6P can induce the conformational changes leading to the disruption of the repressor/DNA complex [9].

T6P dynamics in *E. coli*

To test the utility of our sensors in vivo, we decided to examine the accumulation of intracellular T6P in *E. coli* in both low- and high-osmolarity media. Depending on the osmolarity of the medium, cells are thought to import trehalose using two distinct mechanisms. In low-osmolarity conditions, trehalose is directly phosphorylated to T6P and delivered into the cytoplasm by the phosphotransferase system. T6P is in turn hydrolyzed to glucose and glucose-6-phosphate by the T6P hydrolase TreC. In high-osmolarity conditions, trehalose is hydrolyzed directly to glucose, bypassing T6P [8]. We expressed the T6P sensor T6P-TRACK-20 and the T6P-TRACK-control separately in *E. coli* BL21(DE3). After approximately 45 min of carbon starvation in minimal medium, we administered different amounts of trehalose to equal bacterial suspensions to a final concentration ranging from 0 to 1 mM in the medium. The addition of trehalose caused a positive change in the Venus/eCFP ratio in the cells expressing T6P-TRACK-20, indicating T6P intracellular accumulation. Small fluorescence changes not proportional to the amount of trehalose added were detected in the control samples. Therefore, we subtracted this T6P-independent response from the sensor signal to obtain a corrected FRET response (Fig. 5A).

Intracellular T6P rapidly increases to reach a maximum within 3 min. Moreover, the magnitude of T6P accumulation depends on the extracellular trehalose concentration. These observations suggest that trehalose is directly phosphorylated. The initial boost in T6P is followed by a gradual, seemingly linear decrease. This is caused neither by trehalose depletion nor by photobleaching of the fluorophores. By exclusion, the slow drop in intracellular T6P is likely caused by an increase in catabolic activity downstream of T6P.

Similar regulatory mechanisms, where the initial burst in a metabolite concentration triggers an adaptive response, have been described at the transcriptional level for amino acid synthesis [30]. Finally, we assayed the dynamic response of intracellular T6P under high osmolarity. We cultured cells in LB medium supplemented with either 75 or 250 mM NaCl. After 45 min of carbon starvation in minimal medium, we added 1 mM trehalose. Cells cultivated under salt stress should have lower TreB levels and are expected to show a decrease in Tre uptake through the phototransferase system. Accordingly, the FRET changes observed in cells grown under osmotic stress were largely attenuated (Fig. 5B). Notably, the attenuation could also be caused by increased intracellular trehalose levels that compete with T6P in T6P-TRACK-20. The intracellular T6P measurements match favorably with the known regulatory architecture of the *E. coli* trehalose pathway. Indirectly, these data validate the use of our T6P sensors in vivo in accurately monitoring fast metabolic transients.

T6P dynamics in *S. cerevisiae*

Next, we set out to assess whether the free cytosolic T6P concentration in *S. cerevisiae* is sufficient to differentially inhibit hexokinases in response to the glucose influx. We used a microfluidics/microscope setup to image cells expressing the T6P-TRACK-20 or T6P-TRACK-control sensors and recorded the FRET changes caused by perfusion with different concentrations of glucose (Fig. 6A). Aliquots of cells from an overnight culture in the stationary phase were exposed to step increases in extracellular glucose concentrations ranging from 0.5 to 100 mM. Time-resolved FRET measurements were performed on single cells ($n = 10-20$) and then averaged to provide a population mean. Within seconds after glucose addition, the T6P concentration increased (Fig. 6B) and reached a maximum after 2 to 3 min. Individual cells showed very consistent results, as indicated by the small standard deviations obtained from single cells at any time point (Fig. 6B). Like in *E. coli*, the control sensor showed a small response to glucose addition, but the magnitude of the response did not depend on glucose concentration. The FRET values of the control samples were subtracted from those of the sensor to obtain a corrected FRET response (Fig. 6C). The difference in corrected Venus/eCFP ratio between starvation and after 3 min of glucose supplementation are indicative of the intracellular accumulation of free T6P. Cells expressing the lower affinity T6P-TRACK-190 showed similar results (Fig. 6D). Previous studies have shown that relative FRET changes measured in vivo are often smaller compared with the FRET change measured in vitro with the same sensor protein and an equivalent change in ligand concentration [17,31]. This allowed us to conservatively estimate the in vivo T6P concentration. When using TRACK-190 samples in 10 or 100 mM extracellular glucose, we obtained a corrected FRET change of approximately 8%. We assumed that this in vivo change corresponded to an in vitro change of at least 8%. Based on the in vitro response curve measured for the TRACK-190 sensor (Fig. 3A), we can estimate that on an 8% increase in FRET, the free T6P in the cytoplasm reached at least 140 μ M. This value is a lower bound, in particular considering that the dynamic range of the TRACK-190 sensor spans from 50 to 1000 μ M and that these values are even higher in the presence of trehalose. Regardless of these additional factors, we detected a free T6P concentration that is comparable to the K_i for hexokinase I and II inhibition and, thus, is in line with the hypothesis that T6P can modulate entry of glucose in glycolysis.

Discussion

Cellular regulation occurs at multiple levels with interaction within and between proteins, small molecules, RNA, and DNA. These interactions can feature highly dynamic temporal patterns and rely on precise organization within cellular compartments. The granularity of these events is best captured by experimental methods that preserve cellular structures and allow for continuous measurement, ideally at subcellular resolution. For studies that call for monitoring in vivo levels of metabolites, genetically encoded FRET sensors fulfill these requirements and, thus, are a method of choice. We demonstrated these benefits using a newly developed family of FRET sensors highly specific for T6P. In time-resolved experiments with *E. coli*, we could directly observe the immediate uptake of trehalose by the phototransferase system and the subsequent dynamics suggesting transcriptional adaptation. Using the same reasoning described for the yeast experiments, it was also possible to estimate the intracellular T6P concentration for *E. coli*. We found that the addition of trehalose concentration larger than 0.1 mM trehalose causes an initial burst in the T6P of at least 12 to 15 μM . In addition, the results obtained with osmotic-stressed cells corroborate the model of Tre metabolism previously proposed [8] and clearly confirm the predictions regarding the relation between intracellular T6P levels and the osmolarity of the medium.

In *S. cerevisiae*, we used the new FRET sensors to estimate the free concentration of T6P and shed light on the controversial hypothesis of hexokinase II inhibition. Based on our experiments, the cytosolic T6P concentration in yeast when growing on glucose concentrations above approximately 5 mM should be at least 12 μM . Therefore, it is seemingly high enough to interfere with the kinetics of hexokinase II ($K_i = 40 \mu\text{M}$). Notably, higher T6P concentrations are needed to effectively compete with glucose (1–3 mM [32]) and tangibly inhibit hexokinase activity. Besides, the immediate increase of intracellular T6P in the presence of glucose also contradicts previous speculations that T6P would be effectively channeled within the Tps1–Tps2 complex rather than released to the cytosol [12]. Together, our data support the hypothesis that T6P plays a role in the control in the entry of glucose into glycolysis.

The presented experiments would have been virtually impossible to accomplish with those analytical methods typically used for metabolite analysis (i.e., mass spectrometry and nuclear magnetic resonance) because of disruptive or low-sensitivity measurement. The major caveats for the application of FRET sensors in metabolite reporting are the tedious construction of specific constructs with different affinities, and the heterogeneous Venus/eCFP ratio measured in different hosts in spite of equivalent ligand concentration. A nonbinding control is always necessary to diagnose unspecific effects and eventually correct for biases. However, this correction strategy cannot be applied to single cells. Therefore, accurate measurements of free metabolites at cellular or subcellular resolution is possible only if unspecific effects can be excluded a priori. We expect the T6P–TRACK sensors to become a valuable tool for investigating the role of T6P in more challenging contexts such as plant development and growth [5]. Although trehalose is absent in mammals, it is essential for insects [33] and the sensors can be useful in the search for insecticide compounds targeting the Tre pathway. Because trehalose is similarly important for

nematodes [34], our T6P sensors may serve to screen for drugs targeting the Tre pathway and acting against pathogenic nematodes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig.1. Emission spectra of eCFP-TreR-Venus construct at different T6P concentrations. The Venus band (~530 nm) increases, whereas the eCFP band (~485 nm) decreases, with T6P addition (as indicated by the arrows). Excitation of eCFP was at 430 nm.

Fig.2.

FRET changes of truncated TreR constructs. The bars represent the maximal FRET changes observed in vitro on titration with T6P and are identified with the number of residues that were deleted from the N or C terminus. Error bars represent standard deviations of at least three measurements.

Fig.3.

FRET responses and selectivity of the family of T6P sensors. (A) In vitro titration of sensors and control construct with Hill equation-fitted data (lines) (see Materials and Methods for details). (B) Maximum FRET changes obtained on the addition of up to 10 mM compounds structurally related to T6P or abundant in relevant biological systems. Error bars represent standard deviations of at least three measurements.

Fig.4. T6P titration of T6P–TRACK sensors in the presence of Tre: TRACK-20 (top), TRACK-120 (middle), and TRACK-180 (bottom).

Fig.5. Monitoring T6P levels in *Escherichia coli*. (A) Intracellular levels of T6P caused by the addition of increasing amounts of Tre to the medium (from 0 to 1 mM). (B) Comparison of intracellular T6P levels caused by the addition 1 mM Tre to normal or osmotically stressed cells. Error bars represent standard deviations between three replicates.

Fig.6. T6P in *S. cerevisiae*. (A) Bright field (top) and YFP (yellow fluorescent protein) channel (bottom) microscopy images of yeast cells expressing T6P-TRACK-20. (B) Ratiometric changes of T6P-TRACK-20 (dark gray) and control construct (light gray) on perfusion with 100 mM glucose. (C) Comparison of FRET changes caused by perfusion with increasing concentrations of glucose (values are in mM). The inset plot correlates perfused glucose concentration with the maximum FRET change observed. (D) FRET changes followed by T6P-TRACK-190 on perfusion with 1, 10, or 100 mM glucose.

Table 1

T6P affinity and binding cooperativity of the sensors.

	Dissociation constant (K_d , μM^{-1})	Hill coefficient
T6P-TRACK-20	22 (± 3)	1.4 (± 0.1)
T6P-TRACK-120	119 (± 12)	1.5 (± 0.2)
T6P-TRACK-190	186 (± 14)	1.6 (± 0.1)

Note: Values were determined by fitting in vitro titration data with the Hill equation.