Dynamic analysis of cytosolic glucose and ATP levels in yeast using optical sensors

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Precise and dynamic measurement of intracellular metabolite levels has been hampered by difficulties in differentiating between adsorbed and imported fractions and the subcellular distribution between cytosol, endomembrane compartments and mitochondria. In the present study, genetically encoded FRET (Förster resonance energy transfer)-based sensors were deployed for dynamic measurements of free cytosolic glucose and ATP with varying external supply and in glucose-transport mutants. Moreover, by using the FRET sensors in a microfluidic platform, we were able to monitor in vivo changes of intracellular free glucose in individual yeast cells. We demonstrate the suitability of the FRET sensors for gaining physiological insight by demonstrating that free intracellular glucose and ATP levels are reduced in a hxt5Δ hexose-transporter mutant compared with wild-type and other hxtΔ strains.

Key words: Förster resonance energy transfer (FRET), hexose transport, metabolic flux, microfluidic platform, starvation.

INTRODUCTION

Cellular metabolite levels have predominantly been determined using destructive assays. Such assays can only provide dynamic information when carried out with parallel samples and have limited spatial resolution, i.e. they provide an average for cells in a culture or organ. Isotopes have been used to monitor glucose-transport activity, but they cannot measure glucose uptake in single living cells and interpretations are occluded by metabolism. Thus minimally invasive technologies that can provide real-time information of metabolite levels in a given subcellular compartment at various external supply levels would help to advance our understanding of the mechanisms and control of metabolic fluxes.

For many organisms, including yeast, glucose is the preferred source of energy. In Saccharomyces cerevisiae, glucose uptake is mediated by a family of 17 hexose transporters: Gal2 (galactose transporter 2), Hxt (hexose transporter) 1–11 and Hxt13–17 [1]. These transporters are characterized by broad selectivity for various hexoses and different affinities, thus covering a wide range of external supply levels, with Hxt1–7 being the dominant transporters at typical supply levels of 2% glucose in the medium [2,3]. S. cerevisiae is a facultative fermenter, which, depending on the growth conditions, the type, and the concentration of sugars and/or oxygen availability, will display fully respiratory, fermentative or mixed respiratory/fermentative metabolism [4]. During fermentation, i.e. under anaerobic conditions, cytosolic glucose is oxidized to pyruvate, generating two ATP molecules per glucose molecule. In yeast, ATP levels have been typically measured using bioluminescence assays in cell extracts or NMR [5–7]. To determine the yield of ATP in the cytosol and in mitochondria under various conditions and its relation to cytosolic glucose levels in vivo, a suitable quantitative analytical method for both molecules would be advantageous.

Genetically encoded FRET (Förster resonance energy transfer) nanosensors provide a unique tool enabling dynamic quantification of metabolite analysis with subcellular resolution [8,9]. Genetically encoded FRET-based nanosensors have been developed for a variety of sugars and amino acids. The nanosensors are composed of the bacterial periplasmic binding proteins as a recognition element coupled allosterically to a set of two spectral variants of GFP (green fluorescent protein) as reporter elements [10–13]. Conformational changes induced by ligand-binding to the recognition element are translated into a change in FRET between attached ECFP [enhanced CFP (cyan fluorescent protein)] and EYFP [enhanced YFP (yellow fluorescent protein)] moieties, permitting non-invasive measurements of analyte levels in living cells [11]. To determine analyte levels inside organelles, these genetically encoded nanosensors can be targeted to the respective subcellular compartments, such as nuclei or the endoplasmic reticulum [14,15]. Recently, a new FRET-based ATP sensor that uses the ε subunit of the bacterial F0F1-ATP synthase as a recognition element has been reported [16]. This sensor provides information about the ATP-level changes in the cytosol and mitochondria of individual HeLa cells in response to nutritional changes.

In the present study, we expressed FRET glucose and ATP sensors in yeast and developed a simple fluorimetric assay for measuring ATP-level changes, glucose accumulation rates and steady-state levels of glucose and ATP in yeast cultures using genetically encoded glucose FRET sensors. Furthermore, we implemented the use of a microfluidic platform to monitor glucose accumulation and elimination in individual yeast cells. Consistent with previous work showing induction of the HXT5 gene during starvation [17–19], we found that Hxt5p is the dominant transporter for glucose in starved cells. In agreement with a low level of glucose accumulation in starved hxt5Δ cells, we observed a reduced rate of ATP accumulation in hxt5Δ compared with the wild-type strain.

Abbreviations used: ECFP, enhanced CFP (cyan fluorescent protein); EYFP, enhanced YFP (yellow fluorescent protein), FLIP, fluorescence loss in photobleaching; FRET, Förster resonance energy transfer; GFP, green fluorescent protein; Hxt, hexose transporter; SC medium, synthetic complete medium.

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EXPERIMENTAL

Yeast strains

Yeast strains used in the present study were isogenic to BY4743 [MATα his3Δ1/αhis3Δ1 leu2Δ0/αleu2Δ0 lys2Δ0/αlys2Δ0 met15Δ0/∆0 MET15 ura3Δ0/∆0] (Open Biosystems). Additional yeast strains are described in Supplementary Table S1 at http://www.BiochemJ.org/bj/432/bj4320399add.htm. KY98 was kindly provided by Jos Arens, Swammerdam Institute for Life Sciences, Amsterdam, The Netherlands.

Plasmid constructs

A new sensor FLIPglu-30μ/M13V (FLIP is the fluorescent sensor protein ‘fluorescence loss in photobleaching’) with an affinity for glucose of 30 μM was generated by site-directed mutagenesis of D154A in FLIPglu-170Δ13 (QuickChange®; Stratagene) (primers: 5′-CCGCGGCAATCCGGCTGACAGAGACATCC-3′ and 5′-GGTACGTCCTTCTGACCGCCATGGCCGGG-3′). The affinity of the sensor was determined with affinity-purified protein isolated from Escherichia coli [22] using the unique XhoI and HindIII sites. The plasmid on the backbone of FLIPglu-170n was introduced into pENTR1A (Invitrogen). The nanosensor cassette in FLIPglu-170n was inserted into the BamHI/EcoRV sites of pENTR1A (Invitrogen). The nanosensor cassette in FLIPglu-170n was introduced into pDR1GW-ura3 [23] using Gateway® LR reactions (recombination reactions between attB and attR sites) utilizing pDONR201 as an intermediate vector. Subsequently, different affinities for glucose (2 μM, 30 μM and 600 μM) were generated by site-directed mutagenesis [24] (H152A, D154A and F16A) using mutagenesis primers (F16A, 5′-CTATAGAATCGACGATACGCGGCTGACAGAGACATCC-3′ and 5′-GGTACGTCCTTCTGACCGCCATGGCCGGG-3′). The affinity of the sensor was determined with affinity-purified protein isolated from Escherichia coli [22] using the unique XhoI and HindIII sites. The nanosensor cassette was excised using BamHI and inserted into the BamHI/EcoRV sites of pENTR1A (Invitrogen). The nanosensor cassette in FLIPglu-170n-derived isogenic to BY4743 [MATα his3Δ1/αhis3Δ1 leu2Δ0/αleu2Δ0 lys2Δ0/αlys2Δ0 met15Δ0/∆0 MET15 ura3Δ0/∆0] (Open Biosystems). Additional yeast strains are described in Supplementary Table S1 at http://www.BiochemJ.org/bj/432/bj4320399add.htm. KY98 was kindly provided by Jos Arens, Swammerdam Institute for Life Sciences, Amsterdam, The Netherlands.

Growth conditions and treatments

Yeast cells were grown in YPD medium (10 g/l yeast extract, 20 g/l peptone and 20 g/l dextrose) or in SC (synthetic complete) medium [26] containing 2% glucose (SCαΔ) or 2% maltose (SCαΔ) lacking uracil for selection. For starvation, yeast was incubated either for 16 h in 2% ethanol (SCαΔ) or for 6 h in medium lacking a carbon source (SC−). Cultures were grown at 30°C and harvested in exponential phase (D560 0.6–1.0) for all analyses described.

Growth-monitoring assay

Yeast growth was monitored by light scattering using a microplate reader (Tecan GENiosTM at 595 nm). Selected strains were grown overnight in SCαΔ. Cultures were divided into two portions: one portion was grown in fresh SCαΔ and the other was glucose-starved in SCαΔ; both were diluted to D560 0.05. After 16 h in SCαΔ or SCαΔ, 5 x 106 cells were inoculated into a final volume of 100 μl of SCαΔ in 96-well microplates. Plates were incubated at 30°C with constant shaking; growth was monitored using light-scattering measurements at 20-min intervals.

Fluorimetric analysis of FRET-sensor responses in yeast cultures

For determination of the in vivo response, yeast transformed with the new Venus-series of the Δ13 series FLIPglu sensors [21], FLII12Pglu-700μ/M16 [20,27] or the ATP sensors [16] were grown at 30°C in SCαΔ to D560 0.8–0.9 (CEN.PK2–1C and EBY4000 cells were grown in SCαΔ). Cells were centrifuged (3000 g, 5 min), resuspended and incubated for glucose starvation either in SC−c for 6 h or in SCαΔ for 16 h. Cells were washed in 20 mM Mes, pH 6, then adjusted to D560 0.5 and 180 μl was transferred to a 96-well microplate (Greiner PS, F-bottom) for analysis in a microplate reader (Tecan SafireTM; λex 428/12 nm, λem 485/12 nm and 530/12 nm). Sensor expression in yeast was verified by analysing the emission spectra of CFP (428 nm) and Venus (500 nm). Typically, 42 wells of the microplate were filled with cultures. A row of six wells was filled with cells from the same culture; the first row was filled with non-transformed wild-type cells and three adjacent rows were filled with cultures derived from three wild-type independent transformants using the same plasmid. The next three rows were filled with cultures derived from independent transformants of the mutant to be tested. Emission intensities of CFP and Venus at CFPS were acquired for two cycles (each cycle ~100 s) before addition of 20 μl of glucose. The plate was ejected from the reader, glucose (or another metabolite) was added using a multichannel pipette, then the plate was reloaded and immediately read for eight cycles. Different concentrations of glucose (0–100 mM) were added into each of the six individual wells in a single row. In the present study, the emission ratio is defined as the background-corrected fluorescence intensity at 528 nm divided by the background-corrected intensity at 485 nm. All analyses were repeated (with three biological replicates) at least three times independently. Emission ratios were normalized to the average of the two initial ratio values before glucose addition.

Real-time glucose-accumulation assays

For real-time glucose-accumulation assays, transformed cells were grown in SCαΔ and glucose-starved in SCαΔ. Cells were resuspended in 20 mM Mes, pH 6, to D560 0.5. Ratiometric measurements (Tecan InfiniteTM M200; λex 428/12 nm; λem 485/12 nm and 530/12 nm) were taken until a stable baseline had been established and the time was set to zero. Fluorescence readings were obtained every 20 s. Glucose was injected into individual wells after 20 s (duration 0.2 s) to a final glucose concentration of 50 mM. Subsequently, the procedure was repeated for the next well. Experiments were repeated at least three times independently with three biological replicates.
FRET-imaging analysis for single yeast cells

For single-cell FRET analysis, cells transformed with FLII(Pglu-700) or FLIPgulu-30(Pglu-13V) were grown in SC medium lacking glucose. When transferred to SC medium lacking glucose, the FRET glucose nanosensors were expressed in yeast. To starve cells of glucose, cultures grown in SC medium were transferred to SC for 16 h. Cells were then transferred to microtitre plates and the ratio of Venus/ECFP emission peak intensities were recorded in a microplate spectrophuorimeter before and after addition of glucose. Consistent with the potential of the starved cells to accumulate glucose, the cultures showed sustained concentration-dependent decreases in the Venus/ECFP ratio, indicating accumulation of cytosolic glucose after exposure to extracellular glucose (Figure 1A). Since all four sensors differ in only one or two amino acids in the glucose-binding pockets and show a similar maximal ratio change of ∼1.0 in vitro [21], all four sensors were expected to yield similar maximum ratio changes in vivo, provided that the basal glucose concentration was below the sensor saturation and that uptake was not limiting. Consistent with this prediction, the intermediate affinity sensors FLIPgulu-2(Pglu-13V) and FLIPgulu-30(Pglu-13V) gave maximal in vivo ratio changes of ∼0.3 (note that in vitro and in vivo ratios are not comparable directly due to differences in the conditions; thus the maximal in vitro ratio change of ∼1.0 corresponds to ∼0.3 in vivo). In contrast, FLIPgulu-170(Pglu-13V) and FLIPgulu-600(Pglu-13V) showed reduced responses (Figure 1B). The reduced response of FLIPgulu-600(Pglu-13V) at high levels of added glucose is consistent with its maximum response being limited by saturation of the cell’s uptake systems. The reduced response of FLIPgulu-170(Pglu-13V) suggests that the sensor did not reach its full dynamic range because the sensor concentration exceeded ligand levels, or that before external addition of glucose a fraction of the sensor had bound glucose, e.g. due to low basal glucose levels. If we assume that sensor output is largely unaffected by the in vivo conditions, steady-state cytosolic glucose levels in cells grown in medium containing 2 % ethanol are at or below ∼100 nM (assuming a maximal ratio change of 0.3, an observed ratio change of 0.2 suggests partial saturation; Figure 1B). Intracellular levels could be measured over the full range of the four sensors. The extracellular concentration was 16–46-fold higher relative to the intracellular level when calculated for half-saturation (at the Ks of the respective sensor; Supplementary Figure S1D). The measured cytosolic steady-state levels (at a given external concentration) are a function of the relative rates of uptake and hexokinase-mediated conversion into glucose 6-phosphate. Our results demonstrate that yeast either has the potential to take up glucose before addition of glucose (‘ajar strategy’) or that the uptake activity was induced within minutes after re-exposure to glucose (‘intermediate or induction strategy’).

The approach described above required removal of the microplate from the fluorimeter, creating a time lag between glucose addition and the first measurement. To analyse the response in starved cells immediately after glucose addition, we injected glucose into the wells using a computer-controlled injector and recorded the response instantaneously, or assayed individual cells trapped in a microfluidic device. To increase the signal-to-noise ratio and thus assay sensitivity, the high-sensitivity FRET glucose nanosensor FLII(Pglu-700) was used (Ks 700 μM; note that all four sensors show a comparable ratio change of approx. ∼1.0 in vitro in response to glucose addition [13] (Supplementary Figure S1B) and, assuming a linear detection range between 10 and 90% saturation, report glucose levels over six orders of magnitude (∼20 nM to 6 mM; Supplementary Figure S1C).

RESULTS AND DISCUSSION

FRET glucose sensors covering the nano- to milli-molar range

Owing to technical challenges, conventional methods have provided limited information on steady-state glucose levels in the cytosol of yeast [28]. To measure free-glucose levels in yeast as well as the rate of accumulation and elimination, we developed a simple quantitative detection system using genetically encoded FRET sensors. We had previously constructed FRET glucose nanosensors with affinities of 170 nM, 2 μM and 600 μM. This set of sensors detected glucose levels between 20 nM and 6 μM, but left a gap in the range 20–60 μM [21], a range corresponding to the Km of hexokinase. A new FRET sensor with a Km for glucose of 28.5 μM (FLIPgulu-30(Pglu-13V)) was constructed by mutation of Asp54 to an alanine residue in the MglB glucose/galactose-binding transport protein) recognition element (Supplementary Figure S1A at http://www.BiochemJ.org/bj/432/bj4320399add.htm). To increase sensor robustness, EYFP was replaced by the pH- and chloride-tolerant Venus, producing FLIPgulu-170(Pglu-13V), FLIPgulu-2(Pglu-13V), FLIPgulu-30(Pglu-13V) and FLIPgulu-600(Pglu-13V) [13]. Confocal microscopy

Confocal imaging of yeast cells was performed with a Leica SP5 confocal microscope using a 63x/NA 1.3 glycerol-immersion lens (IMM HCX PL APO; Leica). GFP was excited at 488 nm. Image processing was performed using ImageJ (NIH). To determine the average plot profile, 50 randomly selected cells were analysed and sizes were normalized to the smallest cell in the analysed population.

Cytosolic glucose and ATP levels in yeast

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In vivo measurements for FLIPglu-170n(Δ13V) (Figure 1A). Arrows indicate partial saturation of FLIPglu-170n(Δ13V) due to saturation of uptake systems. (C) Schematic representation of relative responses of FLIPglu-600μ(Δ13V) and FLII12Pglu-700μ(Δ13V) to different sugar analogues at a concentration of 10 mM: glu, glucose; gal, galactose; man, mannose; suc, sucrose; frc, fructose; tre, trehalose; xyl, xylose; Mtl, mannitol; Mal, maltose; Ctrl, control (no sugar added). (A and D) The x-axis is broken to indicate interruption of the time course during glucose addition.

A high-sensitivity FRET sensor for mutant screens

To identify the transporters responsible for the ability of starved yeast cells to accumulate glucose, mutants lacking individual members of the Hxt hexose transporter family (all ten mutants from the stock collection: hxt1Δ, hxt2Δ, hxt3Δ, hxt4Δ, hxt5Δ, hxt8Δ, hxt10Δ, hxt12Δ, hxt14Δ, hxt17Δ) were analysed using the FLII12Pglu-700μ(Δ13V)-based fluorimetric assay system. Although most hxt mutants were unaffected with respect to glucose accumulation, deletion of HXT5 led to a significant reduction in cytosolic glucose accumulation (Figures 4A and 4D).
Given the fact that HXT5 is highly induced, e.g. during the transition from fermentative to glycerol-based respiratory growth [30], and its function as a hexose transporter, the simplest hypothesis is that HXT5 is the dominant uptake system under starvation conditions. The reduced capacity for glucose accumulation of the hxt5Δ strain was confirmed in real-time glucose-accumulation assays and single-cell analyses (Figures 2A and 2B respectively, triangles and grey line). A specific effect of ethanol on Hxt5p activity was excluded, since a comparable reduction in uptake capacity was observed in hxt5Δ/Δ strain starved in a medium lacking a carbon source (Figures 4C and 4D). Thus Hxt5p appears not only to be induced as shown previously [17], but also to dominate glucose accumulation under various starvation conditions. When the hxt5Δ strain was exposed to low amounts of glucose (0.5 mM) before the measurement, no difference to the wild-type was observed (Figures 4E and 4F), suggesting that low amounts of glucose induce other Hxts, which then mask the lack of Hxt5p-mediated glucose uptake activity in the mutant. It is noteworthy that hxt5Δ does not completely lose its ability to accumulate glucose. Also, since the FRET sensors measure the relative rates of uptake and metabolism, the absolute...
profiles of the other or the wild-type (results not shown). Since none of the expression showed no significant differences from the parallel with cells expressing the glucose nanosensor FLII12Pglu-
et al. [16] were expressed in the yeast strain BY4743 and the transferred to microplates. Glucose was added (grey bar) after timepoint 2 at the indicated 700 concentration in wild-type cells was AT P s e n s o r s AT 1.03 (Figures 5A and 5B). The negative-control sensor AT1.03R122K/R126K did not show a significant response after glucose addition (Figure 5C). Yeast cells lacking HXT5 were analysed in parallel. The non-saturating glucose concentration of 0.5 mM resulted in a reduced ratio change in hxt5Δ compared with wild-type for both AT1.03 and AT1.03TEM (Figures 5E and 5F). Thus, not unexpectedly, the reduced ability of hxt5Δ to accumulate glucose limited the availability of cytosolic ATP. For comparison, glucose accumulation was measured in parallel after glucose exposure to the same external glucose concentrations for both wild-type and hxt5Δ (Figures 5D and 5H). Using the equation $[S] = K_d \times (r-r_{\text{min}})/(r_{\text{max}}-r)$ [11], we calculated the cytosolic glucose concentrations after perfusion of the different external concentrations for FLII12Pglu-700μM6 in yeast. [S] represents substrate concentration, $r$ represents the measured ratio ($r_{\text{min}}$ in the absence of glucose, $r_{\text{max}}$ is the maximum ratio observed at the highest glucose level). The cytosolic glucose concentration in wild-type cells was estimated to be 228 μM when cells were exposed to 0.5 mM glucose outside the cells, whereas hxt5Δ cells contained 158 μM under the same conditions. The estimates of the cytosolic glucose levels are based on the assumption that $r_{\text{min}}$ and $r_{\text{max}}$ correspond to the apo and saturated forms of the sensor and that the in vivo environment has not affected the $K_d$ of the sensors. Although the use of a wide spectrum of sensors with differing affinities suggests that this assumption for $r_{\text{min}}$ and $r_{\text{max}}$ is adequate for the glucose measurements, we did not calculate the cytosolic ATP levels since $r_{\text{max}}$ probably had not been reached, thus making an estimate unreliable. Independent in vivo calibration of the sensors will be necessary before they can be used to reliably quantify cellular ATP levels. However, it is obvious that cytosolic ATP levels correlate with increased cytosolic glucose levels in a time-dependent manner, but that ATP levels lag significantly behind the glucose accumulation. The implementation of ATP sensors in yeast will provide a fast assay for changes in the energy status of yeast cells under various growth conditions, e.g. oxygen levels. Furthermore, the FRET sensors can be targeted to mitochondria, as has been performed successfully in mammalian cells to compare cytosolic and mitochondrial ATP levels, with striking results (lower mitochondrial ATP levels compared with cytosolic levels) [16]. Moreover, expressing ATP sensors in yeast mutants would help to elucidate the function of putative energy-related genes and the contribution of glycolysis and the tricarboxylic acid cycle to overall cellular energy status in different growth conditions.

**Increase of ATP levels after glucose resupply is limited in an hxt5Δ strain**

To measure the accumulation of cytosolic ATP levels, the FRET ATP sensors AT1.03 ($K_d$ 3.3 mM), AT1.03TEM ($K_d$ 1.2 mM) and the control sensor AT1.03R122K/R126K developed by Imamura et al. [16] were expressed in the yeast strain BY4743 and the response to addition of glucose to starved cells was measured in parallel with cells expressing the glucose nanosensor FLII12Pglu-700μM6. Yeast cells were grown in SCc and then transferred to SC-c for 5 h. Quantification of FRET from different sensors and culture replicates was performed by determining the ratio of Venus/ECFP emission peak intensities before and after addition of glucose using a microplate spectrofluorimeter. Glucose cellular uptake capacity is most probably underestimated by the assay. Since starved hxt5Δ cells still show residual transport activity, other Hxts, e.g. those not in the knockout collection or with redundant activities, must contribute during starvation. Two double mutants (hxt3Δ/hxt5Δ and hxt6Δ/hxt7Δ) were tested, but showed no significant differences from the hxt5Δ single mutant or the wild-type (results not shown). Since none of the expression profiles of the other HXT genes clustered with HXT5 (results not shown), a systematic analysis of all single mutants and all hxt5Δ/hxtsΔ double mutants will be required to identify the other contributors.

**A specific role of Hxt5p during early growth phase**

The hxt5Δ mutant had been reported to grow slightly more slowly compared with the wild-type after glucose addition to glucose-depleted cells [17]. Also, under the conditions used in the present study for starvation, i.e. transfer from ethanol to glucose medium, hxt5Δ showed delayed half-saturation (567 ± 65 min) compared with the wild-type (405 ± 63 min). Growth curves of microorganisms show a lag phase, controlled by cell-cycle checkpoints that determine the energy status; only when sufficient nutrients are available does cell division proceed. HXT5 transcript and protein levels are low during exponential growth on fermentable carbon sources [17]. Hxt5p protein levels increase during carbon-source depletion [17] and decrease after glucose re-addition [31]. To confirm whether Hxt5p localizes to internal membranes,
or AT1.03R122K of the grey bars indicate the ligand being measured using FRET sensors. Results shown are eight additional cycles (each cycle ∼ 100 s). Labels for glucose (glc) and ATP to the right of the grey bars indicate the ligand being measured using FRET sensors. Results shown are means ± S.D. (n = 6 transformants). The x-axis is broken to indicate interruption of the time course during glucose addition.

Figure 5 Reduced cytosolic glucose accumulation in hxt5Δ mutants correlates with reduced cytosolic ATP accumulation

(A–H) Yeast cells expressing either FLU1\textsuperscript{Δ}Glu-700\textsuperscript{Δ}α6 (A, E), AT1.03 (B, F), AT1.03\textsuperscript{Δ}EMK (C, G) or AT1.03\textsuperscript{Δ}EMK,Δα6 (D, H) were glucose-starved for 5 h in SC−, washed and transferred to microplates. Glucose was added (grey bar) after timepoint 2 at the indicated concentrations (0–10 mM), and time-dependent glucose responses were analysed in wild-type and hxt5Δ. After glucose addition, fluorescence intensities for CFP and YFP channels were measured for eight additional cycles (each cycle ∼ 100 s). Labels for glucose (glc) and ATP to the right of the grey bars indicate the ligand being measured using FRET sensors. Results shown are means ± S.D. (n = 6 transformants). The x-axis is broken to indicate interruption of the time course during glucose addition.

or is present at the plasma membrane under the specific starvation conditions used in the present study, Hxt5p–GFP was localized by confocal microscopy (Supplementary Figure S4A at http://www.BiochemJ.org/bj/432/bj4320399add.htm). Hxt5p–GFP was detected mainly at the plasma membrane. Within 60 min of resupply, plasma-membrane levels of Hxt5p decreased and intracellular levels increased. Within <5 h, Hxt5p levels dropped to almost undetectable levels (Supplementary Figures S4C and S4D), indicating that Hxt5p is specifically used for priming and is then rapidly endocytosed and a suite of other Hxts takes over [32].

Interestingly, overexpression of HXT5 in the hexose-uptake-deficient yeast strain EBY4000 (lacking Gal2 and all the Hxt transporters) [33] using a strong PMA\textsuperscript{Δ} promoter fragment in the pDRf1GW-ura3 plasmid [25] led to a greatly increased transformation efficiency if the cells were incubated for 3 h in ethanol, glucose or maltose (Supplementary Figures SSB–SSD at http://www.BiochemJ.org/bj/432/bj4320399add.htm) compared with direct plating after transformation (Supplementary Figure S5A) on selective-medium plates containing glucose as the sole carbon source. No recovery was needed when transformation reactions were first plated on maltose plates (Supplementary Figure S5F), consistent with the presence of maltose transporters in EBY4000. These findings indicate the need for the presence of an uptake system for immediate glucose accumulation and limited availability of energy for Hxt5p synthesis during the early stages of recovery from starvation or transformation.

The presence of Hxt5p in the absence of external glucose seems counterintuitive, since its production requires energy, which is limiting under starvation conditions, and since the transporter is ‘inactive’ because no substrate is available. Such an expense is only useful if the transporter provides a competitive advantage, e.g. if it allows the cells to take up glucose immediately after new resources become available. Cells with a preformed metabolic pathway can import glucose immediately as an energy source. The earlier the checkpoint requirements are fulfilled, the earlier the organism can divide and grow. In its natural habitat, yeast experiences cycles of extreme sugar supply and subsequent exhaustion. Yeast cells typically are transferred from carbon-poor environments, such as soil, to new habitats rich in nutrients, such as grapes or fruits. \textit{Saccharomyces} is successful in outpacing other microbes also present on a grape or fruit [34].

In summary, we have developed a set of simple assays for dynamic analysis of cytosolic glucose and ATP levels using optical sensors. The use of the sensors allowed exploration of how glucose-starved yeast prepares for future exposure to sugar. FRET sensors allow non-radioactive, simple, cost-effective and rapid analysis of steady-state levels using either fluorimetric assays or FRET imaging of cells trapped in microfluidic devices. The sensors are genetically encoded and can thus be targeted to subcellular compartments such as organelles to provide for the first time information on subcellular levels using a minimally invasive approach [15]. Further analysis using this technology is expected to contribute to a better understanding of the signalling pathways that lead to induction of Hxt5p activity. Moreover, the FRET sensors can be implemented in fermentor technology to monitor the physiological state of the culture in real time in industrial settings [35].

AUTHOR CONTRIBUTION

Clara Bermejo, Farzad Haerizadeh, Hitomi Takanaga and Wolf Frommer designed the experiments. Clara Bermejo, Farzad Haerizadeh, Hitomi Takanaga and Diane Chermak performed the experiments. Clara Bermejo, Farzad Haerizadeh, Hitomi Takanaga, Diane Chermak and Wolf Frommer analysed the data. Clara Bermejo, Farzad Haerizadeh and Wolf Frommer wrote the paper.

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SUPPLEMENTARY ONLINE DATA
Dynamic analysis of cytosolic glucose and ATP levels in yeast using optical sensors
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Figure S1  FRET glucose sensor construct and in vitro and in vivo analysis of glucose levels
(A) FLIPglu-30μA13V construct consisting of ECFP (cyan), mature MglB (red; numbers indicate amino acid positions relative to initiation codon) and Venus (yellow). (B) Glucose-titration curve of FLIPglu-30μA13V. (C) In vitro glucose dose–response curves for the sensors FLIPglu-170nA13V, FLIPglu-2μA13V, FLIPglu-30μA13V and FLIPglu-600μA13V. (D) External glucose concentrations required to bring the cytosolic glucose concentration to a value corresponding to the Kd of the nanosensors were calculated using the response curves in (C). Note that the internal concentrations calculated using nanosensors are at least 15-fold lower than the external concentrations.

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Wild-type yeast cells (BY4743) expressing FLII12Pglu-700δβ6 were grown in SCglc, glucose-starved, washed, resuspended in 25 mM Mes at pH 4.5 (A), pH 6 (B) and pH 8 (C) and transferred to microplates. Glucose in Mes buffer at the pH indicated was added after timepoint 2 at 0 and 100 mM concentrations and time-dependent FRET changes were monitored. After addition of glucose to the cells, fluorescence intensities in the CFP and YFP channels were measured for eight additional cycles (each cycle ~100 s). Results shown are means ± S.D. (n = 6 transformants).
Figure S3  Effect of pH on the in vivo sensor response measured in individual yeast cells

Wild-type yeast cells (BY4743) expressing FLI12Pglu-700x46 were grown in SC\(^g\), glucose-starved, washed, resuspended in 25 mM Mes, pH 6, trapped in a microfluidic device, washed again with Mes, pH 6, and exposed to pulses of 25 mM Mes at various pH values with (blue shading) or without (unshaded) 100 mM glucose (glc). Time-dependent glucose responses were analysed, including dead-volume correction as described in the Experimental section in the main paper. (A) Average response of 30 cells; results shown are means ± S.D. (B) Response of a single cell.
Figure S4  Quantification of the HXT5–GFP localization

KY98 cells containing an integrated version of a translational HXT5–GFP fusion [17] were starved for 16 h in SCeth. Cellular localization of Hxt5p was studied using confocal microscopy. Images were taken at zero time (A) and 1 h (B) and 5 h (C) after glucose resupply. Scale bar, 10 μm. (D) The distribution of Hxt5p in cross-sections was quantified using Plot Profile (ImageJ) for 50 cells in each condition. Cell sizes were normalized to the smallest cell analysed. Blue diamonds represent yeast cultures starved of carbohydrate for 16 h. Red squares represent yeast cultures starved for 16 h and resupplied with glucose for 5 h.

Figure S5  Requirement of Hxt activity during recovery from transformation

EBY.VW.4000 was transformed with the plasmid pDR[HXT5]. Transformed cells were plated on to selective medium containing glucose as the sole carbon source (A). Plating was performed after recovery for 3 h on medium containing SCeth (B), SCglc (C) or SCmal (D). (E) Negative control: the empty vector pDRf1GW-ura3. (F) Positive control: EBY.VW.4000 transformed with pDRf1GW-ura3 and plated on to SCeth.
## Table S1  List of yeast strains used in the present study

<table>
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<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
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<td>CEN.PK2-1C</td>
<td>MATα ura3-52 leu2-3,112 trp1-289, his3Δ1 MAL2-8c   SUC2</td>
<td>Eckhard Boles, Institute of Molecular Biosciences, Goethe University Frankfurt, Frankfurt, Germany</td>
</tr>
<tr>
<td>EBY.VW4000</td>
<td>MATα ura3-52 leu2-3,112 trp1-289 his3Δ1 MAL2-8c SUC2 hxt17 hxt13 hxt15 hxt16 hxt14 hxt12 hxt9 hxt11 hxt10 hxt8 hxt54 hxt2 hxt57 gal2 sst1 gpt1 ydr247y K60c</td>
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<td>WAY.78-1</td>
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<td>[2]</td>
</tr>
<tr>
<td>ENY.WA-1A</td>
<td>MATα ura 3-52 his3Δ1 leu2-3,112 trp1-289 MAL2-8c MAL3 SUC3</td>
<td>[2]</td>
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<tr>
<td>KY98</td>
<td>MATα MAL2-8C SUC2 HXT5::GFP</td>
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### REFERENCES


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