Determination of in vivo kinetics of the starvation-induced Hxt5 glucose transporter of Saccharomyces cerevisiae

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Abstract

We have investigated the role and the kinetic properties of the Hxt5 glucose transporter of Saccharomyces cerevisiae. The HXT5 gene was not expressed during growth of the yeast cells in rich medium with glucose or raffinose. However, it became strongly induced during nitrogen or carbon starvation. We have constructed yeast strains constitutively expressing only Hxt5, Hxt1 (low affinity) or Hxt7 (high affinity), but no other glucose transporters. Aerobic fed-batch cultures at quasi steady-state conditions, and aerobic and anaerobic chemostat cultures at steady-state conditions of these strains were used for estimation of the kinetic properties of the individual transporters under in vivo conditions, by investigating the dynamic responses of the strains to changes in extracellular glucose concentration. The \(K_m\) value and the growth properties of the HXT5 single expression strain indicate that Hxt5 is a transporter with intermediate affinity.

Keywords: Saccharomyces cerevisiae; Glucose transport; Sugar uptake; Hxt5; Starvation; Kinetic analysis

1. Introduction

Transport of glucose across the plasma membrane into the cell is the first step of glucose metabolism. Saccharomyces cerevisiae can deal with extremely broad ranges of glucose concentrations and glucose can be metabolized effectively at concentrations from higher than 1.5 M down to micromolar concentrations [1]. This implies the presence of a complex and highly regulated glucose uptake system.

The major glucose transporters of S. cerevisiae are encoded by genes HXT1–4 and HXT6–7 [2–4]. All of them are regulated by the kind and concentration of the carbon source [5]. Moreover, the kinetic parameters of the transporters as determined by zero trans-influx measurements with radioactively labelled glucose vary considerably, and the \(K_m\) values for glucose have been determined to reach from 1.1 mM for Hxt7 to about 100 mM for Hxt1 [6,7].

Deletion of all these genes together resulted in a strain nearly unable to take up glucose and to grow on it as the sole carbon source under laboratory growth conditions [8,9]. However, also all of the other yeast genes from HXT8–17 (except for HXT12) and HXT5 are able to mediate uptake of hexoses if overexpressed [10]. These observations suggest that most of the yeast Hxt proteins are able to transport hexoses but that some of them are only used under specific conditions and for specific purposes.

One of the hexose transporters in yeast whose physiological role is not yet clear is the Hxt5 protein. Recently, it has been suggested that Hxt5 may serve as a ‘reserve’ transporter in the initial uptake of glucose, when glucose is absent and becomes available again [7]. Most studies concerning microbial processes have analyzed cell metabolism during growth of the organisms. In their natural environment, however, microorganisms spend most of their time under conditions of no or very slow growth. Slow growth or stationary phase is usually due to severe limitation or starvation for several or specific nutrients. Here, we report that the HXT5 gene becomes strongly expressed during starvation conditions.
In all previous studies, the kinetic parameters of glucose uptake have been investigated by zero trans-influx measurements with radioactively labelled glucose. This method uses yeast cells incubated in phosphate buffer, i.e. under in vitro conditions [6,7]. However, it is not clear how those conditions might influence the properties of the hexose transporters. Therefore, it is desirable to develop a method to study glucose uptake under in vivo conditions. Here, we describe a new approach to assess the kinetic properties of glucose transporters during growth of the yeast. We used the dynamic response to changes in extracellular glucose concentration of yeast strains expressing only individual hexose transporters for the estimation of their kinetic properties in vivo. With this approach we first confirmed the kinetic properties of Hxt1 and Hxt7 as measured under in vitro conditions. We then determined Hxt5 to be an intermediate-affinity glucose transporter in vivo.

2. Materials and methods

2.1. Yeast strains and growth conditions

All yeast strains described in this work were derived from strain CEN.PK2-1C (MATa leu2-3,112 ura3-52 trpl-1 289 his3-D1 MAL2-8 8 Suc2) or from the isogenic strain CEN.PK113-5D (MATa ura3-52 MAL2-8x2 Suc2). The construction of the yeast strain EBY.VW4000 (MATa Δhxt1-17 Δgal2 Δαgt1 Δmph2 Δmph3 Δtst1 leu2-3,112 ura3-52 trpl-289 his3-D1 MAL2-8x2 Suc2) carrying multiple deletions of its hexose transporter genes (HXT) was described in [10]. Rich media were based on 1% yeast extract and 2% peptone (YP), supplemented for auxotrophic requirements as described [11]. Minimal media consisted of 0.17% Difco yeast nitrogen base without amino acids and ammonium (YNB), supplemented for auxotrophic requirements and with 0.5% (NH4)2SO4 and various carbon sources. Yeast cells were grown aerobically at 30°C on a rotary shaker or on agar plates. Cell growth was monitored by measuring optical densities at 600 nm (OD600) or comparing growth of yeast colonies on agar plates.

2.2. Construction of yeast strains

2.2.1. Genomic HA-tagging of HXT5

Three consecutive copies of a hemagglutinin (HA) epitope were fused in the genome to the carboxy-terminal end of the HXT5 open reading frame (ORF) by using a modification of the PCR targeting technique [11]. First, the two primers HATAG-1 and HATAG-2 (Table 1), which contain a 16-bp complementary sequence at their 3' ends, were used in a PCR reaction to amplify each other. The resulting 134-bp fragment was cleaved with XhoI and SaII restriction sites in front of the first loxP site of plasmid pUG6, resulting in plasmid pUG6-HA. This plasmid was then used as a template to generate by PCR, with primers J1-HX5HA and J2-HX5HA (Table 1), a DNA molecule consisting of a 3xHA-kanMX marker cassette flanked by short homology regions to the end of the HXT5 locus. The 1.7-kb PCR product was transformed into strain CEN.PK113-5D, selecting for resistance to G418 (200 mg l−1) on YPD agar plates, and used to replace the stop codon of HXT5 by the 3xHA-kanMX cassette. Thereby, the triple HA-epitope was fused in frame to the carboxy-terminal end of Hxt5, allowing to detect it with commercially available anti-HA antibodies. After transformation of this strain with plasmid pSH47, the kanMX marker was removed as described [11], resulting in strain JBY20. Yeast transformation was as described [12].

2.2.2. Genomic HXT5 promoter-lacZ fusion

Genomic replacement of the HXT5-1-4 gene cluster in strain CEN.PK113-5D by a PCR-amplified lacZ-kanMX reporter cassette was used to fuse the promoter and the first 48 nucleotides of the truncated HXT5 ORF to the Escherichia coli lacZ gene [13]. For PCR amplification of the lacZ-kanMX reporter cassette, plasmid pUG6lacZ [13]

Table 1

<table>
<thead>
<tr>
<th>Primers used in this work</th>
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<tr>
<td>HATAG-1</td>
</tr>
<tr>
<td>5'-GCGCGCTTCATGATCTCTGAAAGTTCCAGAATCTGGTCCTACCCTTGTACGCTGTACGTGTJ-3'</td>
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<tr>
<td>HATAG-2</td>
</tr>
<tr>
<td>5'-CCTCCTGCAACTTGTAACAATATGTTGATCTTACACTACATAGAAGAAGTAGACGCTACGTGATCGTJ-3'</td>
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<tr>
<td>J1-HX5HA</td>
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<td>5'-GATCCGAGACCTTATTTTAATAAGATTGATCTTACACTACATAGAAGAAGTAGACGCTACGTGATCGTJ-3'</td>
</tr>
<tr>
<td>J2-HX5HA</td>
</tr>
<tr>
<td>5'-GACATCTGGATCAGTGTAATAATGTTGATCTTACACTACATAGAAGAAGTAGACGCTACGTGATCGTJ-3'</td>
</tr>
<tr>
<td>SI-HX5SH</td>
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<td>5'-GCTGTTGAGATCAGTCAAGCTTCTCAACTGCTTCTCAACTGAGAATAAGAAGTAGACGCTACGTGATCGTJ-3'</td>
</tr>
<tr>
<td>S2-HX5SH</td>
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<td>5'-GATCCGAGACCTTATTTTAATAAGATTGATCTTACACTACATAGAAGAAGTAGACGCTACGTGATCGTJ-3'</td>
</tr>
<tr>
<td>PROHXT7-1</td>
</tr>
<tr>
<td>5'-GAGACTGTAGATCTGTGTAGACJ-3'</td>
</tr>
<tr>
<td>PROHXT7-2</td>
</tr>
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<td>5'-GAGACAGTGATCTGTGTAGACJ-3'</td>
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<tr>
<td>INTPH1-1</td>
</tr>
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<td>5'-GCTTCTGCAAGCTGAGCCACTTTCTCTAGAATAAGCTCGGGCGCGCGCTGTTAGACGJT-3'</td>
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<tr>
<td>INTPH2-1</td>
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<td>5'-GAAGACCCTTCTGTCGCGCTGTTAGACGJT-3'</td>
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<td>C1-HX7T1</td>
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<td>5'-CAAGAGAATTGACAGTGGAGGCTGTTAGACGJT-3'</td>
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<td>C4-HX7T1</td>
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<td>C4-HX7H5</td>
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<td>5'-CAAGAGAATTGACAGTGGAGGCTGTTAGACGJT-3'</td>
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and the primers S1-LH514 and S2-HX514 (Table 1) were used.

2.2.3. HXT1, 5 and 7 single expression strains

To determine the kinetic properties of Hxt5 and to compare them with those of Hxt1 and Hxt7, the HXT1, 5 and 7 genes were constitutively expressed in strain EBY.VW4000 which is deleted for all its hexose transporters and unable to take up hexoses [10]. For this, a 392-bp HXT7 promoter fragment was integrated into the genome of strain EBY.VW4000, into the former HXT3-6-7 gene cluster region, by using a modification of the PCR targeting technique, resulting in a very strong and constitutive HXT7 promoter-terminator expression cassette [14]. Part of the HXT7 promoter together with part of the HXT7 coding region from −392 to +30 was amplified by PCR with primers PROHXT7-1 and PROHXT7-2 (Table 1), and plasmid p21-PST [8] as a template. The PCR product was cleaved with SpeI at both ends and cloned in the correct orientation into the SpeI site of plasmid pUG6 behind the second loxP site, resulting in plasmid pUG6-kPHXT7. This plasmid was then used as a template to generate by PCR, with primers INTPH7-1 and INTPH7-2, a DNA molecule consisting of a kanMX-HXT7p marker cassette flanked by short homology sequences to the HXT7 promoter (−770 to −720) and HXT7 terminator regions. The 2.4-kb PCR product was transformed into strain EBY.VW4000 (whose HXT3-6-7 gene cluster is replaced by a loxP site), resulting for resistance to G418 (200 mg l−1) on YPMaltose agar plates, and used to replace the HXT3 promoter−loxP region by the kanMX-HXT7p cassette. After transformation with plasmid pSH47, the kanMX marker was removed as described [11], resulting in strain EBY.VW4002.

The HXT1 gene was amplified by PCR from plasmid pHXT1-1 [6] with primers C1-IHXT1 and C4-IHXT1 (Table 1). The HXT7 gene was amplified by PCR from plasmid p21-PST with primers C1-IHXT7 and C4-IHXT7. Both PCR products containing short homology sequences to the HXT7 promoter−terminator expression cassette of strain EBY.VW4002 were transformed into this strain, selecting for growth on YPGlucose agar plates. Integration into the genomic expression cassette by homologous recombination resulted in strains JBY01 (HXT1+) and JBY02 (HXT7+). To construct an FOA (5-fluoroorotic acid)-counterselectable marker expression cassette in strain EBY.VW4002, the Kluyveromyces lactis URA3 ORF was PCR-amplified from strain MS7-62 (gift of C. Falcone, Rome, Italy) with primers I-KURA31 and I-KURA32 (Table 1), resulting in a DNA fragment with the KlURA3 ORF flanked by short homology regions to the HXT7 promoter and HXT7 terminator. The DNA fragment was transformed into strain JBY02 selecting for growth on a synthetic medium without uracil and with maltose as the carbon source, resulting in strain JBY15 containing a genomically integrated HXT7 promoter(−392/+1)_KlURA3−HXT7 terminator expression cassette.

The HXT5 gene was amplified by PCR with plasmid p10 [8] and primers C1-IHXT5 and C4-IHAH5 (Table 1), resulting in a DNA fragment containing the HXT5 ORF fused to an HA epitope sequence and flanked by short homology regions to the HXT7 promoter and HXT7 terminator. This DNA fragment was transformed into strain JBY15, selecting for growth on synthetic me-

Fig. 1. Induction of Hxt5 expression during nitrogen starvation. The strain JBY20 containing an HA-tagged HXT5 allele was grown in YP medium with 3.5% rafinose into the exponential growth phase. After washing the cells once in YNB medium without carbon and nitrogen source, the cells were shifted to YNB medium with 5% glucose and without nitrogen source. At the indicated times, cells were harvested, membrane fractions were prepared, and the proteins were subjected to Western analysis with anti-HA antibodies.
medium with 0.2% glucose and 1 mg ml⁻¹ FOA. The resulting strain expressing an Hxt5–HA fusion protein behind the strong HXT7 promoter fragment was named JBY19.

2.3. Preparation of membrane fractions and Western blot analysis

Preparation of yeast membrane fractions and Western blot analysis were done essentially as described in [15]. Anti- HA high-affinity antiserum (Roche) was diluted 1:10,000. Sheep anti-rat peroxidase conjugate (Roche), diluted 1:1000. Anti-HA high-affinity antiserum (Roche) was diluted above. The composition of the synthetic mineral medium was balanced for a biomass concentration of 2.5 g DW l⁻¹ as described above with the exception of the glucose concentration in the feeding medium, which here was 30 g glucose l⁻¹.

2.4. Fed-batch cultivation

The different mutants of S. cerevisiae (HXT single expression strains JBY19 (HXT5⁺), JBY02 (HXT7⁺), JBY01 (HXT1⁺) and CEN.PK2-1C (wild-type)) were grown in fed-batch culture under glucose (wild-type) or maltose (mutants) limitation at 30°C in a stirred tank bioreactor (Biostat E, Braun Biotech International, Melsungen, Germany). A controlled exponential feeding rate led to a constant specific growth rate (μ) of μ^SP = 0.1 ± 0.009 h⁻¹:

\[ F = \left( \frac{\mu^{SP}}{Y_{X/S}} + m_S \right) \left( \frac{c^0_{V_0}}{V_0} \right) \frac{c^0_s}{c^0_s} e^{(\mu T')P4 0.009 h^{-1}} \]  

with \( c^0_X \), \( V_0 \) being the initial biomass concentration and liquid volume, respectively; \( c^0_s \) the concentration of glucose/maltose in the feed = 65 g l⁻¹; \( m_S \) the maintenance coefficient = 0.04 h⁻¹ [16], \( Y_{X/S} \) the biomass yield = 0.44 g dry weight (g DW) (g glucose)⁻¹ and \( Y_{X/S} \) = 0.4 g DW (g maltose)⁻¹ (the yield coefficients were determined in the first fed-batch experiments). The outflow was kept at 1 vvm and pH was controlled at 5.0 by addition of 2 M NaOH. The oxygen concentration was not allowed to decrease under 40% of air saturation. Maltose was used as sole source of carbon and energy to guarantee standardized growth rates independent of the different glucose transporters.

The concentrations of the components in the synthetic mineral medium were precalculated from element balancing [17] for production of 15 g DW l⁻¹ as described in [18]. The bioreactor was connected to an exhaust air analysis device consisting of a paramagnetic oxygen analyzer (Oxygor 6N) and an infrared CO₂ analyzer (Unor 6N), both from Maihak (Hamburg, Germany). For data acquisition and feeding of glucose or maltose during the fed-batch operation the bioreactor was connected to a personal computer equipped with a data acquisition device (Burr Brown PCI 20098C-1, Intelligent Instrumentation, Filderstadt, Germany).

2.5. Chemostat cultivation

2.5.1. Aerobic cultivation

S. cerevisiae JBY19 (HXT5 single expression strain) was grown in continuous chemostat culture (D = 0.1 ± 0.002 h⁻¹) under glucose limitation at 30°C in a stirred tank bioreactor (KLF2000, Bioengineering, Wald, Switzerland) with a working volume of 1.5 l. The pH control and foam prevention was carried out as described above. The composition of the synthetic mineral medium was balanced for a biomass concentration of 15 g DW l⁻¹ as described above, with the exception of the glucose concentration in the feeding medium, which here was 30 g glucose l⁻¹.

2.5.2. Anaerobic cultivation

S. cerevisiae JBY19 (HXT5 single expression strain) was also grown in anaerobic continuous chemostat culture (D = 0.07 ± 0.002 h⁻¹) under glucose limitation at 30°C in a stirred tank bioreactor (laboratory glass fermenter 2 l, Hans W. Schmidt, Mainz, Germany) with a working volume of 1.5 l. The pH control and foam prevention were carried out as described above.

The composition of the synthetic mineral medium was balanced for a biomass concentration of 2.5 g DW l⁻¹ as described above with the exception of the glucose concentration in the feeding medium which was 25 g glucose l⁻¹ in these experiments. For anaerobic growth ergosterol, oleic acid and Tween 80 was added as described in [18].

2.6. Glucose pulse experiments

To determine the kinetic parameters of the individual mutants, pulse experiments were carried out. Starting from steady-state or quasi steady-state (fed-batch) conditions a pulse of concentrated glucose was injected into the bioreactor with a syringe. Samples were taken using a vacuum-driven sampling device in combination with rapid cryofiltration (−20°C) [19].

Glucose was assayed by the glucose dehydrogenase (EC 1.1.47) method using the Granutest 250 test kit (Merck, Darmstadt, Germany) and mutarotase. Ethanol was determined with GC (type 5890, Series II with FID-detector, Hewlett Packard, Avondale, IL, USA; column: FS-OV1-1-CB, Chromat. Service, Langerwehe, Germany) using 1-butanol as internal standard.

2.7. Kinetic analysis

For estimation of the kinetic parameters, measured glucose concentrations were compared with the numerical integration of the dynamic balance equation, for the fed-batch processes:

\[ \frac{d c_S}{d t} = \frac{F}{V_F} (c^0_S - c_S) - \frac{c^0_{c_S}}{K_m + c_S} c_S \]
and for the chemostats:

\[ \frac{dS}{dt} = D(C^0_S - C_S) - \frac{q_{\text{max}}}{K_m + C_S} C_S \]

Integration was performed with the numerical integrator ACSL (Version 11.5.3, Advanced Continuous Simulation Language, Mitchel and Gauthier). For estimation of the kinetic parameters the optimization package OptdesX (Version 2.0.4, Design Synthesis, Simulated annealing algorithm) was used. Minimization of the identification functional

\[ J(P) = \sum_{k=1}^{N} \left( \frac{C_S^{\text{max}}(t_k) - C_S^{\text{pred}}(t_k)}{C_S^{\text{meas}}(t_k)} \right)^2 \]

(\(t_k\) being the time of sampling, \(N\) the number of samples) leads to the estimates of \(K_m\) and \(q_{\text{max}}\). Errors were less than 10%.

3. Results and discussion

3.1. The HXT5 protein appears during nitrogen starvation

Genome-wide transcriptional analyses using DNA microarrays and Northern blot experiments have previously shown that the yeast HXT5 gene is repressed by glucose, and is induced by glucose depletion, by aerobic growth in glucose-limited steady-state chemostat cultures, by osmotic shock, during growth on non-fermentable carbon sources and during sporulation [7,20-23]. To analyze the expression profile of Hxt5 at the protein level, a triple HA epitope sequence was fused in frame to the end of the genomic HXT5 ORF by using a modification of the PCR targeting technique [11]. Cells of this strain (JBY020) were grown in YP medium with 3.5% raffinose into the exponential growth phase, and Western blot analysis with HA-specific antibodies was used to detect the Hxt5 protein in a membrane-enriched fraction of yeast cell extracts. No Hxt5 protein was detectable under such conditions (Fig. 1). However, after shifting the yeast cells to a medium without any nitrogen source, the amount of Hxt5 protein increased considerably during 6 h (Fig. 1).

3.2. The HXT5 gene is induced under starvation conditions

To analyze whether this strong increase in the amount of Hxt5 protein during nitrogen starvation is due to transcriptional induction, a genomic replacement of the HXT5-1-4 gene cluster in strain CEN.PK113-5D by a PCR-amplified lacZ-kanMX reporter cassette was used to fuse the promoter and the first 48 nucleotides of the truncated HXT5 ORF to the E. coli lacZ gene. Measuring of β-galactosidase activities revealed that the HXT5 promoter is strongly induced by nitrogen starvation. During exponential growth in YNB medium with 3.5% glucose β-galactosidase activities were 4 mU (mg protein)\(^{-1}\), whereas they reached 706 mU (mg protein)\(^{-1}\) 24 h after a shift of the cells to YNB medium with 3.5% glucose and without any nitrogen source. Moreover, the HXT5 promoter turned out to be strongly induced also by carbon starvation: 24 h after a shift of the yeast cells to YNB medium with ammonium sulfate and without any carbon source, β-galactosidase activities reached 556 mU (mg protein)\(^{-1}\) compared to 4 mU (mg protein)\(^{-1}\) during exponential growth in YNB medium with 3.5% glucose.

3.3. Construction and growth of HXT single expression strains

To determine the kinetic properties of Hxt5, the HXT5 gene was constitutively expressed behind an HXT7 promoter fragment in the genome of strain EBY.VW4000 which is deleted for all its hexose transporters and unable to take up hexoses [10]. For a strong and constitutive expression, an HXT7 promoter\(^{\text{orf}}\)-\(\text{terminator}\) expression cassette was first integrated into the genome of strain EBY.VW4000 (Δhxt7). The cassette was modified into an FOA-counterselectable marker expression cassette by integrating the K. lactis URA3 gene. Yeast strains expressing a functional URA3 gene cannot grow in the presence of FOA. This strain could be used to integrate the HXT5 ORF behind the HXT7 promoter fragment after transformation and homologous recombination selecting for growth on a glucose medium containing FOA. Similarly, the HXT1 and the HXT7 ORFs were also individually expressed behind the HXT7 promoter fragment in the same background and compared to the HXT5 single expression strain.

Growth of the various strains was compared on YNB agar plates supplemented with different kinds and concentrations of carbon sources (Fig. 2). Whereas no differences in growth between the strains could be seen on a maltose medium, the EBY.VW4000 parent strain did not grow on any media containing glucose as a carbon source, irrespective of the concentration [10]. As compared to the other strains, the HXT1 strain (JBY01) showed only slow growth on a 5 mM glucose medium (Fig. 2). In contrast, with glucose concentrations between 100 and 250 mM, growth was similar to that of the wild-type strain. This is a consequence of the very low affinity (\(K_m\) glucose \(\approx\) 100 mM) of the Hxt1 glucose transporter [6]. Growth of the HXT7 and HXT5 strains (JBY02 and JBY19) was comparable to that of the wild-type strain on all different glucose concentrations (Fig. 2), confirming the high/intermediate affinity of the transporters for glucose [6,7].

3.4. Kinetic characterization of the glucose transport mutants

The kinetics of glucose uptake were compared between the wild-type strain and the individual HXT single expres-
In previous studies, the kinetic parameters of glucose uptake have been investigated by zero trans-influx measurements with radioactively labelled glucose and with yeast cells incubated in phosphate buffer, i.e. under in vitro conditions [6,7]. In this study, the kinetic parameters of different glucose transporters were estimated by investigating the dynamic response of HXT single expression strains to changes in extracellular glucose concentration under in vivo conditions. For this purpose, a concentrated glucose solution was injected into carbon-limited yeast fed-batch cultures at quasi steady-state conditions. Samples were taken from the bioreactor using a vacuum-driven sampling device and extracellular glucose was measured enzymatically after a rapid cryofiltration step. For estimation of the kinetic parameters measured glucose concentrations were compared with the numerical integration of the dynamic balance equation. Minimization of an identification functional led to the estimates of $K_m$ and $q_{max}$. The implicit assumption of this approach is that glucose transport has the overwhelming control of the consumption under the experimental conditions of interest. This assumption is, first of all, supported by several indications from the literature [24]. The important hypothesis is furthermore sustained by a comprehensive approach, consisting of the dynamic modelling of the central metabolism of S. cerevisiae under aerobic [19,25,26] and anaerobic [27,28] growth conditions. The dynamic model rests upon the experimental observation of intracellular metabolites in response to a stimulus in the form of the extracellular glucose concentration. The most important results of this analysis are summarized in Fig. 3, showing the hierarchy of flux control coefficients for sugar uptake in response to fractional increases of the enzyme activities involved in the path from glucose to ethanol. The flux control coefficients were estimated from the dynamic model according to the approach suggested by Mauch et al. [29]. Since only a subset of the flux control coefficients is shown in Fig. 3 (excluded, for example, are the coefficients of enzymes in-
volved in the pentose phosphate shunt), flux control coefficients of this subset do not necessarily sum up to one. From this hierarchy of sensitivities it can be concluded that the enzyme responsible for the transport of glucose via the cell membrane exerts the overwhelming control strength on the flux. Nevertheless, it cannot be totally excluded that addition of glucose to the cultures causes a slight shift of the flux control to the glycolytic enzymes downstream of the transporter.

The described experiments and estimations of the kinetic parameters were carried out with the HXT single expression strains JBY19 (HXT5\(^\text{+}\)), JBY02 (HXT7\(^\text{+}\)), JBY01 (HXT1\(^\text{+}\)) and CEN.PK2-1C (wild-type). Fig. 4 shows the comparison of the time courses for extracellular glucose after a pulse of glucose for the different strains. Already at first glance, the pronounced differences in the shape of the curves reflecting the transient response seem to support the well-known distinction between the high-affinity (Hxt7) and low-affinity (Hxt1) transporter. From an initial data inspection, Hxt5 appears to be a transporter with intermediate affinity.

Applying the parameter estimation procedure described above provided the following \(K_m\) values for the high-affinity transport systems in CEN.PK2-1C (wild-type) and single expression strain JBY02 (HXT7\(^\text{+}\)):

\[
(K_m)_{\text{HXT7}} = 1.1 \text{ mM}
\]

and

\[
(K_m)_{\text{wild-type}} = 0.4 \text{ mM}
\]

The value obtained for the single expression strain JB02 (HXT7\(^\text{+}\)) is in excellent agreement with the \(K_m\) values reported in the literature [6], which were obtained with the aid of zero trans-influx measurements. This result may also serve as a further support for the hypothesis of the overwhelming control of glucose flux through the transporter and justify the strategy of the simple stimulus–response method. An interesting phenomenon is, however, related to the lower \(K_m\) value for the wild-type compared to that of the single expression strain JB02 (HXT7\(^\text{+}\)). A possible explanation can be found from the investigations of Petit et al. [30] showing that the Hxt2 transporter is also expressed under glucose-limited growth conditions. For the hexose transporter Hxt2 a \(K_m\) value of 10 mM for cells growing on high glucose concentrations has been estimated, and biphasic uptake kinetics with a high-affinity component (\(K_m=1.5\) mM) after growing on low glucose concentrations [6]. Therefore, the affinity of Hxt2 seems to be modulated in dependence on the external glucose concentration. This lower \(K_m\) value may mask the slightly higher value of the high-affinity transporter Hxt7 expressed under glucose-limited growth conditions during fed-batch cultivation. A slight discrepancy, however, remains regarding the \(K_m\) value obtained for the wild-type strain as compared to data from the literature. The \(K_m\) value for various wild-type strains identified from zero trans-influx measurements is in the range of

\[
0.5<(K_m)_{\text{wild-type}}<1.0 \text{ mM}
\]

[4,6,31]. Most of these measurements have been performed with samples from shake-flask experiments. The physiological conditions of such experiments are not well defined. It is particularly difficult to consider the role of the Hxt2 transporter for which a modulation of its activity by growth conditions has been clearly indicated [30].

Further investigations into the identification of the kinetic properties of the Hxt5 transporter were performed during continuous cultivation of the single expression strain. Starting from steady-state conditions at \(D=0.1\) \pm 0.002 h\(^{-1}\) a pulse of glucose was injected to give an initial bulk concentration of 10 g l\(^{-1}\). These experiments
were performed under aerobic and anaerobic growth conditions. Fig. 5 shows the comparison of measured and computed glucose concentrations during the transient conditions at aerobic growth. The following parameters were estimated by minimizing the error square:

\[ (q_s)_{\text{in situ}} = \frac{D (c_S - c_0)}{c_X} \]  \hspace{1cm} (5)

and compared with the results of uptake rates estimated from the kinetic expression

\[ (q_s)_{\text{kinetics}} = \frac{q_s^{\text{max}} (c_S)_{\text{steady state}}}{K_m + (c_S)_{\text{steady state}}} \]  \hspace{1cm} (6)

with the measured steady-state glucose concentrations \((c_S)_{\text{steady state}} = 258 \text{ mg l}^{-1}\) for aerobic and \((c_S)_{\text{steady state}} = 230 \text{ mg l}^{-1}\) for anaerobic growth. The calculations yielded the following results:

**Aerobic:**

\[ (q_s)_{\text{in situ}} = 1.7 \text{ mmol g DW}^{-1} \text{ h}^{-1} \]

\[ (q_s)_{\text{kinetics}} = 1.4 \text{ mmol g DW}^{-1} \text{ h}^{-1} \]

**Anaerobic:**

\[ (q_s)_{\text{in situ}} = 3.6 \text{ mmol g DW}^{-1} \text{ h}^{-1} \]

\[ (q_s)_{\text{kinetics}} = 1.5 \text{ mmol g DW}^{-1} \text{ h}^{-1} \]

It has been claimed that the efflux of glucose due to internal accumulation of glucose during the zero trans-influx measurement causes a lower uptake rate [22]. The reasonable agreement between the uptake rates observed under aerobic growth conditions suggests that such effects of internal glucose concentrations are of minor importance in the time span of measurements of glucose concentrations after the glucose pulse. The pronounced difference under anaerobic conditions has been previously observed by Diderich [22]. The lower value of glucose uptake rate estimated from the kinetic measurements prompts the hypothesis that the increased influx of glucose in response to the glucose pulse under anaerobic conditions causes a traffic jam in the glycolysis and leads to an internal accumulation of glucose for a longer time period. Ongoing research focuses on the measurement of intracellular glucose concentrations under the same experimental conditions to get further insight into these problems.

4. Conclusions

Previous investigations had indicated that the \(HXT5\) glucose transporter gene of \(S. cerevisiae\) is a silent gene and is not transcribed during ‘normal’ laboratory growth conditions [8,9]. However, more recent data had shown that \(HXT5\) is induced during glucose depletion or stress conditions [7,22,23,32]. Here, we have demonstrated that indeed the \(HXT5\) gene is induced by starvation. Both, nitrogen starvation and carbon starvation trigger a strong induction of \(HXT5\) transcription and a pronounced increase in the amount of Hxt5 protein. In their natural environment yeast cells spend most of their time under severe nutrient limitation or starvation. Therefore, the transcriptional regulation points to an important role of Hxt5 for glucose uptake under starvation and stress conditions. It is remarkable that other transporters like Hxt1, Hxt6 and Hxt7 are proteolytically degraded under the same conditions, at least during nitrogen starvation [15,33,34]. Moreover, Hxt5 turned out to be a transporter with a moderately high affinity for glucose. Therefore, it is well suited for uptake of glucose irrespective of its concen-
tration. Consistent with this, expression of only Hxt5 enabled the yeast cells to grow like wild-type cells at any glucose concentration, in contrast to expression of the low-affinity transporter Hxt1. Thus, Hxt5 seems to be used by the yeast cells as a major glucose transporter (i) during nitrogen starvation or other stress conditions such as osmotic shock in order to provide glucose for energy production or (ii) during glucose limitation or carbon starvation in order to enable the cells to rapidly take up glucose as soon as it becomes available again.

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