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Fsy1, the sole hexose-proton transporter characterized in *Saccharomyces* yeasts, exhibits a variable fructose:H^+ stoichiometry

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ABSTRACT

In the model yeast *Saccharomyces cerevisiae*, hexose uptake is mediated exclusively by a family of facilitators (Hxt, hexose transporters). Some other *Saccharomyces* species (e.g. *Saccharomyces bayanus* and *Saccharomyces pastorianus*) possess, in addition, a specific fructose transporter (Fsy1, fructose symporter) that has been previously described to function as a proton symporter. In the present work, we compared growth of a yeast strain in which *FSY1* occurs naturally in anaerobic, fructose- and glucose-limited chemostat cultures. Especially at low specific growth rates, fructose-proton symport was shown to have a strong impact on the biomass yield on sugar. We subsequently employed energized hybrid plasma membrane vesicles to confirm previous observations concerning the mode of operation and specificity of Fsy1 mediated transport. Surprisingly, these experiments suggested that the carrier exhibits an unusual fructose:H^+ stoichiometry of 1:2. This energetically expensive mode of operation was also found consistently *in vivo*, in shake flask and in chemostat cultures, and both when Fsy1 is the sole transporter and when the Hxt carriers are present. However, it is observed only when Fsy1 is operating at higher glycolytic fluxes, a situation that is normally prevented by downregulation of the gene. Taken together, our results suggest the possibility that fructose symport with more than one proton may constitute an energetically unfavorable mode of operation of the Fsy1 transporter that, in growing cultures, is prevented by transcriptional regulation.

1. Introduction

Hexose uptake may proceed through a variety of mechanisms that include facilitated diffusion, proton symport and Na^+ symport, the first two being very commonly found in fungi [1]. In yeasts belonging to the phylum *Saccharomycotina*, a large family of hexose facilitators included in the major facilitator superfamily (MFS) plays a dominant role [2]. This family is represented in the model organism *Saccharomyces cerevisiae* by the Hxt 1–17 transporter proteins, the two glucose sensors Snf3 and Rgt2 and the galactose transporter encoded by the *GAL2* gene [3]. The recent availability of an increasing number of genome sequences has unveiled a multitude of homologs in other yeasts and revealed a particularly dynamic evolutionary history for this family of proteins [4].

In *Saccharomyces cerevisiae* and in four additional, closely related *Saccharomyces* species, glucose and fructose transport proceeds solely through facilitated diffusion mediated by the Hxt proteins [2]. However, in *Saccharomyces uvarum* and *Saccharomyces eubayanus*, as well as in the hybrids *Saccharomyces pastorianus* (lager brewing yeast) and *Saccharomyces bayanus*, an additional transporter is present which is specific for fructose and catalyzes transport *via* a proton symport mechanism [5–7]. Cloning and sequence analysis of the *S. pastorianus FSY1* gene encoding this symporter, showed that it belongs to the sugar porter sub-family of the MFS but is only distantly related to the Hxt proteins [6]. The *S. pastorianus FSY1* gene is identical to that present in its parent species *S. eubayanus* while whole genome sequence of *S. uvarum* CBS 7001, showed that it harbors a different allele whose level of divergence is consistent with the phylogenetic distance between the two species [7,8].

Abbreviations: MFS, major facilitator superfamily; Hxt, hexose transporters; Fsy1, fructose symporter; PMF, proton motive force; CL–PMV, cytochrome *c* oxidase liposome–plasma membrane vesicles; FCCP, Carbonyl cyanide p-[trifluoromethoxyl]-phenyl-hydrazone; TMPD, *NNN′N′*-tetramethyl-p-phenylenediamine; TPP, tetraphenylphosphonium

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Expression of *FSY1* in an *S. cerevisiae* genetic background devoid of hexose transporters, confirmed that it exhibits a high affinity for fructose (K_m~0.16 mM; [6]) and a somewhat lower affinity for sorbose. It is, however unable to transport measurable amounts of glucose, which is a highly unusual feature among hexose transporters [6].

In *S. cerevisiae*, metabolism and regulatory effects of glucose and fructose are very similar. Both hexoses are taken up by the same transporters [3,9] and two of the three hexose kinases accept both sugars as a substrate [10]. Moreover, the ability of fructose to trigger signaling through e. g. the main glucose repression and the Ras/PKA pathways parallels that of glucose, although it may in some cases elicit a less vigorous response [11,12]. As a consequence, growth parameters are very similar when *S. cerevisiae* is grown on either glucose or fructose as carbon and energy source.

The main objective of the present work was to characterize the kinetics and energetics of Fsy1-mediated fructose transport and to evaluate the impact of fructose-proton symport *via* Fsy1 on the physiology of yeast cells. To this end, anaerobic, fructose- and glucose-grown chemostat cultures were performed, and an *in vitro* system consisting of yeast plasma membrane vesicles in which a stable proton motive force can be created was employed to quantitatively analyze proton coupling of Fsy1-mediated fructose transport.

2. Materials and methods

2.1. Strains and growth conditions

S. bayanus PYCC 4565 and *S. pastorianus* PYCC 4457 (Portuguese Yeast Culture Collection (PYCC), Caparica, Portugal) naturally harbor the Fsy1 transporter and were used for chemostat experiments. *S. cerevisiae hxt-null* EBY VW4000 [3], *S. cerevisiae* I3 (EBY VW4000 *ura3::FSY1*) [5] and I4 (prototrophic version of *S. cerevisiae* I3) were used to study transport stoichiometry. *S. cerevisiae* strain I4 was constructed by transforming I3 with amplicons encompassing the *HIS3*, *LEU2* and *TRP1* coding sequences, resulting in a prototrophic strain, more amenable to use in chemostat experiments.

For shake flask cultivation, yeast strains were grown at 30 °C in YNB medium (w/o amino acids) containing the indicated concentrations of carbon source and supplements.

2.2. Chemostat culture

Anaerobic chemostat cultures were performed in a 2 L ADI bioreactor (Applikon Dependable Instruments) with a working volume of 1 L. Effluent was removed from the middle of the culture *via* an Applikon electrical level controller. This set-up ensured that biomass concentrations in the effluent line differed by less than 1% from those in samples taken directly from the culture. Cultures were conducted under carbon and energy limitation on a mineral medium supplemented with vitamins, trace elements and the anaerobic growth factors ergosterol and Tween-80, as described by Weusthuis et al. [13]. Glucose or fructose (25 g/L), and the supplements were added to the medium after separate sterilization. Cultures were performed at 25 °C, and at a stirrer speed of 750 rpm. pH was maintained at 4.5 by automatic addition of 2 M KOH, *via* an Applikon ADI-1020 biocontroller. To ensure anaerobic conditions, the reactor and the reservoir vessel were flushed with nitrogen gas at a flow rate of 0.5 L/min. The dissolved-oxygen tension of the culture was continuously monitored with an oxygen electrode (Ingold) and was below 0.1% air saturation.

Aerobic carbon limited chemostat cultures were performed in a 1 L Biolab bioreactor with a working volume of 500 mL. Synthetic medium containing vitamins and trace elements solution was prepared as described by Verduyn et al. [14] and a concentration of 4 g L^{-1} fructose was used.

2.3. Analytical procedures

Sugar concentrations in the culture supernatants were determined using either commercial kits, namely, the glucose oxidase assay (Merck) for glucose and the glucose/fructose assay (Boehringer) for fructose. Ethanol and glycerol were quantified by HPLC with an Aminex HPX-87H column (Bio-Rad), using an ERMA ERC-7515A refractive index detector. This HPLC set-up was also used to measure glucose and fructose concentrations in chemostat reservoir media. Ethanol concentrations in culture supernatants were corrected for ethanol concentrations in the reservoir vessels (originating from addition of ethanolic stock solutions of ergosterol). Gas efflux from the fermenter was cooled to 2 °C in a condenser and dehydrated in a Perma Pure Dryer (PD-625-12P) before quantification using an IR detector model 864 (Beckman) for CO_2 and an oxygen analyzer (Taylor Servomex Co,) for O_2. Gas efflux rate was measured accordingly to Weusthuis et al. [15] and specific rates of CO_2 production were calculated as described by Van Urk et al. [16].

The dry weight of the cultures was determined using a microwave oven and 0.45-μm-pore-size filters [17].

2.4. Preparation of cytochrome c oxidase liposome–plasma membrane vesicles (CL–PMV)

Plasma membranes were isolated from *S. cerevisiae* I3 and *hxt-null* strains exponentially growing on mineral medium containing 30 mM fructose according to the procedure described by Van Leeuwen et al. [18]. The purified membranes were resuspended in 10 mM Tris, 1 mM EDTA, pH 7.4 and stored in liquid nitrogen.

Cytochrome *c* oxidase was isolated according to Yu et al. [19]. Proteoliposomes containing purified cytochrome *c* oxidase were prepared in potassium phosphate buffer at pH 5.5 from purified commercial *Escherichia coli* phospholipids by a dialysis procedure [18], and stored in liquid nitrogen.

Yeast plasma membrane vesicles and the proteoliposomes were mixed in 50 mM potassium phosphate buffer, 1 mM $MgCl_2$, pH 5.5 at a 1:20 protein/phospholipids ratio and fused by freezing in liquid nitrogen, followed by slow thawing at room temperature. The thawed suspension was sonicated (2×5 s) in an ultrasound bath to obtain hybrid vesicles.

2.5. Measurement of internal volume and proton motive force across plasma membrane vesicles

For estimation of the internal vesicle volume, 1 μCi of D-[U-^{14}C] glucose was incorporated in the vesicles during the fusion procedure. The vesicles were separated from the medium by filtration, washed on the filters and the radioactivity was counted, as indicated in Section 2.7 for the transport assays. An internal volume of 0.4 μL/mg phospholipids was determined [18].

The transvesicular pH gradient (ΔpH) was measured according to Clement and Gould [20], using pyranine. Hybrid vesicles with incorporated pyranine were suspended in 2 ml of 50 mM potassium phosphate, 1 mM $MgCl_2$ at pH 5.5, and the energization of the system was achieved by the addition of the electron donor and mediators: 100 μM *NNN′N′*-tetramethyl-p-phenylenediamine (TMPD), 10 μM cytochrome *c* and 10 mM ascorbate.

The membrane potential (Δψ) was inferred from the distribution of the lipophilic cation tetraphenylphosphonium (TPP^+) by using a TPP^+-selective electrode [21]. Hybrid vesicles were suspended in 2 ml of 50 mM potassium phosphate, 1 mM $MgCl_2$, and 2 μM TPP^+, at pH 5.5. The system was energized as described above. Δψ was calculated as previously reported [22,23], assuming symmetric binding.

2.6. Determination of the percentage of vesicles with fructose carrier

To evaluate the percentage of vesicles containing the fructose carrier, the uptake of fructose, under non-energized conditions, was compared in hybrid vesicles prepared from a *S. cerevisiae hxt-null* mutant (empty vector control strain lacking a functional fructose carrier) and I3 strain. Discrimination between hybrid vesicles with and without functional carriers was enabled by the much faster initial uptake of the substrate until equilibrium in vesicles containing the carrier [24]. Based on the difference between the two distinct fructose uptake curves, about 23% of the hybrid vesicles were estimated to contain active fructose transporters. Calculations on fructose uptake assays were therefore based on the assumption that 23% of the vesicles contained the fructose carrier.

2.7. Transport assays

2.7.1. Measurement of radioactive fructose accumulation

Hybrid vesicles were incubated at 30 °C with 15 μM of D-[U-^{14}C] fructose (320 mCi/mmol) for 5 min. The reaction was initiated by addition of the electron donor system described in Section 2.5 and stopped by dilution with 2 ml ice-cold 100 mM LiCl. The mixture was immediately filtered through 0.45-μm pore-size cellulose nitrate membranes (Whatman) and the filters were washed with 2 ml of ice cold 100 mM LiCl and placed into 6 ml scintillation liquid (OptiPhase HiSafe 2, PerkinElmer, Inc.) for radioactivity determination. Non-specific fructose adsorption to the filter was determined in an assay in which D-[U-^{14}C]fructose was added dilution of the vesicles with cold LiCl solution.

2.7.2. D-[U-^{14}C]fructose transport assays in CL–PMV

Hybrid vesicles were incubated at 30 °C in the presence of the electron donors indicated above for 4 min to fully energize the membranes. The reaction was started by the addition of D-[U-^{14}C]fructose (specific activities between 11.4 and 320 mCi/mmol) at the desired concentration and stopped after 1 min by dilution with 2 ml ice-cold 100 mM LiCl. The mixture was immediately filtered through 0.45-μm pore-size cellulose nitrate membranes (Whatman) and the filters were washed with 2 ml LiCl and placed into 6 ml scintillation liquid for radioactivity determination. The non-specific fructose adsorption to the filter was determined in an assay in which D-[U-^{14}C] fructose was added after dilution of the vesicle suspension with LiCl.

2.7.3. D-[U-^{14}C]fructose transport assays in intact cells

Initial rates of D-[U-^{14}C]fructose (Amersham) transport were determined in 5 s assays as previously described [25]. D-[U-^{14}C]fructose specific activities used were 0.5 mCi/mmol for fructose concentrations below 2 mM and approximately 1.8 mCi/mmol for higher fructose concentrations.

2.7.4. H^+ symport assays

Initial uptake rates of sugar/proton symport activity were determined by computer recording the alkalinization of an aqueous yeast cell suspension upon fructose addition using a standard pH meter [26] and home designed software (four pH values recorded per second).

The kinetic data obtained were fitted to a one- or two-component Michaelis–Menten kinetic model, using GraphPad Prism software.

2.7.5. Statistics

Paired samples Student's t-tests were used to compare specific ethanol and CO_2 production rates between fructose and glucose limited chemostats at low dilution rates ($D \leq 0.075\ h^{-1}$; Fig. 1C and Supplementary Fig. 1). A p value < 0.05 was regarded as statistically significant. Statistical analysis was performed using IBM SPSS statistics (version 20) software.

3. Results and discussion

3.1. Impact of fructose transport via Fsy1

S. bayanus PYCC 4565 naturally harbors the *FSY1* gene [5]. This strain is a hybrid containing genomic DNA of at least two closely related species, *S. eubayanus* and *S. uvarum*. Many *S. bayanus* strains also carry some genomic DNA from *S. cerevisiae* [7], but this has not been established for strain *S. bayanus* PYCC 4565. A previous study on transcriptional regulation of the *FSY1* gene in strain PYCC 4565 showed that it is induced at low fructose or glucose concentrations (below 5 mM) and repressed at higher concentrations of both hexoses [5]. The *FSY1* allele in *S. bayanus* PYCC 4565 is identical to that originally isolated from *S. pastorianus* PYCC 4457 and to the allele present in the wild species *S. eubayanus* [7] but is slightly different from the *S. uvarum* CBS 7001 allele (91% identity). We found that the transporters encoded by the two different *FSY1* alleles are functionally and biochemically indistinguishable (results not shown), so that as far as can be judged presently, the presence of either allele has the same physiological consequences.

To evaluate the impact of the presence of an active fructose transporter operating simultaneously with the Hxt facilitators in *Saccharomyces*, *S. bayanus* strain PYCC 4565 was cultivated in chemostat at a range of dilution rates between 0.025 and 0.15 h^{-1} and using either glucose or fructose as carbon and energy source. In order to better assess the effect of the energetic burden posed by active fructose transport, the cultures were performed under anaerobic conditions so that metabolism would be exclusively fermentative. If, as has been demonstrated for *S. cerevisiae* [13] the proton stoichiometry of the plasma membrane ATPase in *S. bayanus* equals one and, moreover, Fsy1 has a fructose–H^+ stoichiometry of one, fructose transport *via* Fsy1 would lead to a maximum ATP yield of 1 mol ATP (mol fructose)$^{-1}$, as compared to 2 mol ATP (mol fructose)$^{-1}$ in fermentative cultures grown on glucose, which is transported *via* Hxt transporters. Simultaneous involvement of Fsy1 and Hxt transporters in fructose uptake should lead to intermediate ATP yields.

Residual sugar concentrations in anaerobic, fructose- and glucose-limited chemostat cultures of *S. bayanus* PYCC 4565 were strongly dependent on dilution rate which, in steady-state chemostat cultures, is equal to the specific growth rate (Fig. 1A). At dilution rates above 0.08 h^{-1}, residual fructose concentrations in the fructose-limited cultures increased above 5 mM, a concentration that was previously shown to lead to transcriptional repression of the *FSY1* gene [5]. At these higher dilution rates, where transport of fructose and glucose is therefore expected to be completely dependent on Hxt transporters, residual sugar concentrations in the fructose-limited chemostat cultures were much higher than in the glucose-limited chemostat cultures (Fig. 1A). This observation probably reflects the higher affinity of Hxt transporters for glucose, as compared to their affinity for fructose [9]. Hence, while operation of Fsy1p ensures efficient fructose uptake at low dilution rates, at higher dilution rates the glucophilic nature of the Hxt carriers becomes evident due to the virtual absence of Fsy1 mediated high affinity fructose transport.

At the highest dilution rates tested, biomass yields of the glucose- and fructose-limited chemostat cultures were the same, indicating that there were no additional energy costs associated with fructose fermentation (Fig. 1B). In contrast, at the lowest dilution rate tested (0.025 h^{-1}), the biomass yield on fructose was 31% lower than that on glucose (Fig. 1B and D). Assuming a fructose–H^+ stoichiometry of one, this reduction is less than the 50% reduction that would be expected when fructose would be completely transported *via* Fsy1 and indicates that, at this low specific growth rate, still about 40% of fructose transport occurs *via* facilitated diffusion. These observations demonstrate that the increased affinity for fructose that is enabled by the involvement of Fsy1 in its transport has a major impact on growth energetics in anaerobic cultures.

As a consequence of the lower ATP yield from fructose fermentation at these low specific growth rates ($D \leq 0.075\ h^{-1}$), specific rates of alcoholic fermentation ($p = 0.026$) and CO_2 production ($p = 0.017$) were significantly higher in the fructose-limited chemostat cultures than in the glucose-limited cultures (Fig. 1C and Supplementary Fig. 1A). In anaerobic, glucose-limited chemostat cultures of *S. cerevisiae*, specific rates of glycerol production are linearly correlated with specific growth rate [27], consistent with the role of glycerol as a redox sink for NADH [28]. Specific rates of glycerol production were therefore not expected to be affected by the mode of sugar transport. Indeed, glucose- and fructose-limited, anaerobic chemostat cultures of *S. bayanus* PYCC4565 showed virtually the same linear relationships between dilution rate and specific rate of glycerol production (Supplementary Fig. 1B). We did not observe marked differences in the protein content, profiles of minor metabolites (pyruvic, succinic, fumaric and lactic acid) or morphology of glucose- and fructose-grown cultures (data not shown).

3.2. Fsy1 mediated transport in hybrid vesicles

In order to get more insight in the biochemical properties of Fsy1 mediated fructose uptake, we subsequently set out to characterize this transporter *in vitro* and in conditions that avoid interference of other carriers. This was achieved by using the *S. cerevisiae* recombinant strain I3, which is devoid of the complete set of Hxt related transporters and expresses the *FSY1* gene from *S. pastorianus* PYCC 4457. In accordance with the specificity of the carrier, this strain is unable to grow on glucose and growth on fructose relies entirely on uptake through Fsy1 [5].

This strain was used as a source of purified plasma membranes that were incorporated in vesicles containing a proton motive force (PMF) generating system. To this end, vesicles were prepared by fusion of purified yeast plasma membranes from *S. cerevisiae* strain I3 with purified *E. coli* phospholipids. This type of vesicle has been previously used to characterize the active galactose transporter in *Kluyveromyces marxianus* [18] and the maltose/H^+ symporter in *S. cerevisiae* [29]. A stable PMF is generated by incorporation of cytochrome *c* oxidase in the vesicles. When inserted in the vesicular membrane in the correct orientation and in the presence of reduced cytochrome *c* in the extravesicular medium, this enzyme functions as a proton pump generating a stable proton electrochemical gradient, inside negative and alkaline, that can be used to drive an active transport system. The cytochrome *c* oxidase liposome–plasma membrane vesicle (CL–PMV) preparation exhibited a stable PMF of −100 mV, calculated by measuring separately the electrical membrane potential and the delta pH (Supplementary Fig. 2A and B), which is sufficient to allow for significant sugar accumulation. In addition, it could be established that 23% of the vesicles possess a fructose symporter (Supplementary Fig. 2C). This value is in between those obtained for vesicles containing the galactose symporter from *K. marxianus* (11%, [18]) and the maltose symporter from *S. cerevisiae* (30%, [29]).

Energization of Fsy1p-containing CL–PMV incubated in the presence of 30 μM D-[^{14}C]-fructose led to a six-fold accumulation of fructose inside the vesicles (Supplementary Fig. 2D). This level of accumulation is lower than that observed for the maltose/H^+ symporter in *S. cerevisiae* [29]. The dependence of Fsy1 activity on the two components of the proton motive force was determined separately. Energizing the vesicles in the presence of FCCP (a protonophore uncoupler) prevented D-[^{14}C]-fructose accumulation, while addition of FCCP to D-[^{14}C]-fructose loaded vesicles triggered a rapid efflux of the labeled sugar (Supplementary Fig. 2D). Subsequently, ionophores were used to dissipate separately the proton gradient without disturbing membrane potential (nigericin, which enables K^+/H^+ antiport) and to eliminate the membrane potential without affecting the proton gradient (using valinomycin, a K^+ ionophore). As previously observed, the effect of valinomycin is stronger than the effect of nigericin, although the latter also causes a clear decrease in D-[^{14}C]-fructose accumulation in energized CL–PMV (Supplementary Fig. 2C). As previously postulated [30], the pronounced effect of valinomycin may indicate that it not only dissipates the membrane potential but also directly inhibits transport.

The Fsy1 transporter has been previously reported to transport fructose and sorbose, but not glucose [6]. This substrate specificity

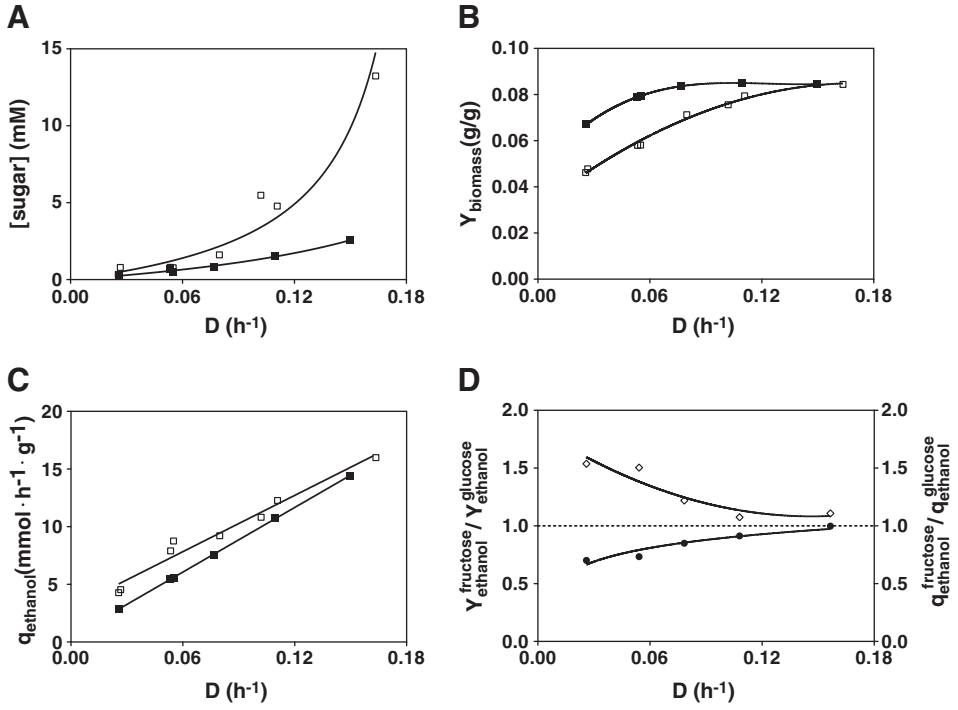

Fig. 1. Anaerobic chemostat cultures of *Saccharomyces bayanus* 4565. Effect of the dilution rate (D) on the residual sugar concentration (panel A), biomass yield (panel B) and specific ethanol production rate (panel C) on fructose (□) or glucose (■) limited anaerobic chemostat cultures of *S. bayanus* PYCC 4565 (pH 5, 25 °C). Panel D: effect of dilution rate on the biomass yield ratio (●) and specific ethanol production rate ratio (◊) calculated from each of the fructose and glucose limited chemostat cultures.

was confirmed by the addition of non-radioactive sugars at a concentration of 30 mM to energized, D-[^{14}C]-fructose loaded CL–PMV. Addition of fructose or sorbose elicited rapid efflux of labeled fructose, while glucose had no detectable effect (Fig. 2).

The K_m of D-[^{14}C]-fructose uptake determined using fructose concentrations between 0.03 mM and 0.750 mM was 0.53 mM, which is in good agreement with the value determined for intact cells of strain I3 (K_m ~ 0.44 mM, Fig. 3), from which the plasma membranes were purified. This suggests that the heterologous lipid environment in the hybrid vesicles does not seriously affect the kinetics of fructose transport *via* Fsy1.

As shown in Table 1 the stoichiometry of fructose/H^+ symport mediated by Fsy1, calculated from the value of the PMF and the fructose chemical gradient was 0.49, which indicates that Fsy1 translocates two protons per fructose molecule. This is in marked contrast to, for example, the maltose/H^+ symporter in *S. cerevisiae*, which only transports a single proton. Such an unfavorable energetic balance for sugar transport has to our knowledge not been reported in fungi and prompted us to investigate the proton stoichiometry of Fsy1 transport *in vivo*.

3.3. Fructose:H^+ stoichiometry in vivo

The *in vivo* fructose:H^+ stoichiometry of Fsy1 mediated transport was first examined in *S. cerevisiae* I3, which was the source of the plasma membranes incorporated in the vesicles. This was done by measuring D-[^{14}C]-fructose uptake velocities and the rate of H^+ influx (see Sections 2.7.3 and 2.7.4, respectively, for a description of the methods) simultaneously for each sample of cells. *S. cerevisiae* I3 was cultivated under the same conditions as those used for plasma membrane isolation (shake flask culture, in YNB medium supplemented with 30 mM fructose). The results, depicted in Fig. 3, were consistent with the fructose:H^+ stoichiometry of 1:2 calculated from experiments with CL–PMV, suggesting that these did not originate from an artifact associated with the *in vitro* system.

S. cerevisiae I3 was unable to grow in YNB medium containing 60 mM fructose as sole carbon and energy source when the cultures were supplemented with antimycin A, an inhibitor of respiration (result not shown). This would be expected if 2 protons are symported for each fructose, as this would lead to a net ATP yield of zero from the alcoholic fermentation of fructose. However, a prototrophic derivative of *S. cerevisiae* I3 (named I4) cultivated in aerobic fructose limited chemostat at increasing dilution rates showed a fructose:H^+ stoichiometry of 1:1 at lower dilution rates, that nevertheless increases to up to 1:3.6 at the highest dilution rate (Fig. 4).

In *S. cerevisiae* I4, the heterologously expressed Fsy1 transporter operates in a nonnatural context. We therefore sought to study stoichiometry in the unaltered context of the transporter, *i.e.* in a strain

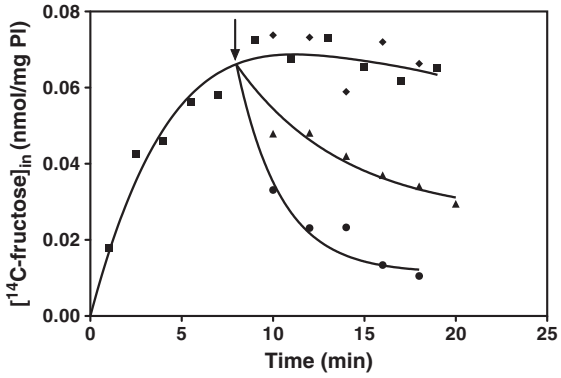

Fig. 2. Specificity of Fsy1 mediated transport in hybrid vesicles. D-[^{14}C] fructose uptake (■) in *Saccharomyces cerevisiae* I3 hybrid membrane vesicles, at pH 5.5. Unlabelled fructose (30 mM; ●), sorbose (30 mM; ▲) or glucose (30 mM; ♦) were added 8 min after energization with ascorbate, TMPD and cytochrome *c*.

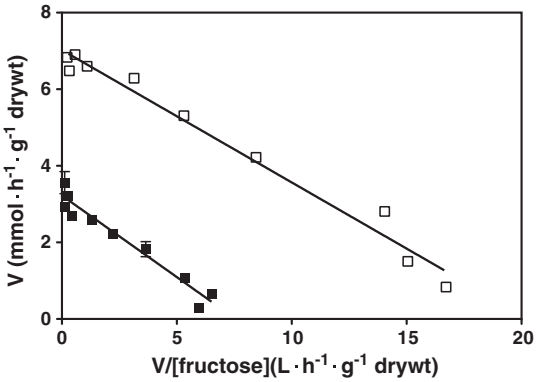

Fig. 3. Kinetics of Fsy1 mediated fructose and proton uptake *in vivo*. Eadie–Hofstee plot of the initial uptake rates of D-[^{14}C]fructose (■) and proton influx (□) by *Saccharomyces cerevisiae* I3 at pH 5.5 (K_m ~ 0.44 mM).

that naturally expresses the *FSY1* gene and which contains a normal complement of Hxt proteins. To this end, *S. pastorianus* PYCC 4457 was grown in aerobic fructose-limited chemostat at different dilution rates (Supplementary Fig. 3). The *FSY1* gene was originally isolated from this strain which, like other lager strains, is supposed to be a hybrid between *S. eubayanus* and *S. cerevisiae* [31]. In this case, fructose is transported both by Fsy1 and by the Hxt proteins, and biphasic transport kinetics can be observed (Supplementary Fig. 3). To determine stoichiometry, the V_{max} of the high affinity component considered to be that of Fsy1 mediated transport, was compared with the V_{max} calculated for H^+ influx, which should be independent of the activity of Hxt facilitators. Also for this strain, the proton stoichiometry of Fsy1 appeared to vary according to the dilution rate. While a stoichiometry of 1:1 was observed for the lowest dilution rates, it reached 1:2 at dilution rates above 0.13 h^{-1}. This is in line with our observations in a *S. bayanus* strain that also naturally harbors the *FSY1* gene (results not shown) and in *S. cerevisiae* I4 (Fig. 4).

We conclude that the variable proton stoichiometry of Fsy1 does not seem to be an artifact associated with the context in which Fsy1 operates (natural *vs* heterologous setting, presence *vs* absence of the Hxt proteins). It is presently difficult to foresee what the trigger for the switch in stoichiometry might be. It seems to be unrelated to extracellular pH because this parameter is kept constant during chemostat cultivation, but it could be somehow linked to changes in the glycolytic flux. Since the 1:2 stoichiometry was observed both *in vivo* and *in vitro* for strain I3 we cannot exclude the possibility that it involves a modification undergone by the Fsy1 protein itself. In what concerns the physiological role of the stoichiometry change, we considered the possibility that higher stoichiometries might be allowed to operate when the relative contribution of Fsy1 mediated fructose uptake is relatively low when compared with the contribution of Hxt mediated facilitated diffusion, because under these circumstances it would not represent a serious burden for the cell. Such conditions are observed when the glycolytic flux is high. A regulated adjustment of the stoichiometry to 1:1 might then take place at lower glycolytic fluxes when the relative contribution of Fsy1 to overall fructose transport is expected to be most significant. However, it should be kept in mind that higher glycolytic fluxes are normally associated with higher extracellular sugar concentrations, and under those circumstances the *FSY1* gene is repressed. This would in

Table 1
Relationship between PMF and fructose accumulation in CL–PMV at pH 5.5. All values were determined at the steady-state sugar accumulation. Fructose accumulation concerns only the vesicles containing a fructose carrier.

$\Delta\psi$ (mV)	ZΔpH (mV)	PMF (mV)	$\Delta\mu_{frutose}/F$ (mV)	η_{ap}	Fructose/H^+ stoichiometry
−37.3	61.4	98.7	48.5	0.49	1:2

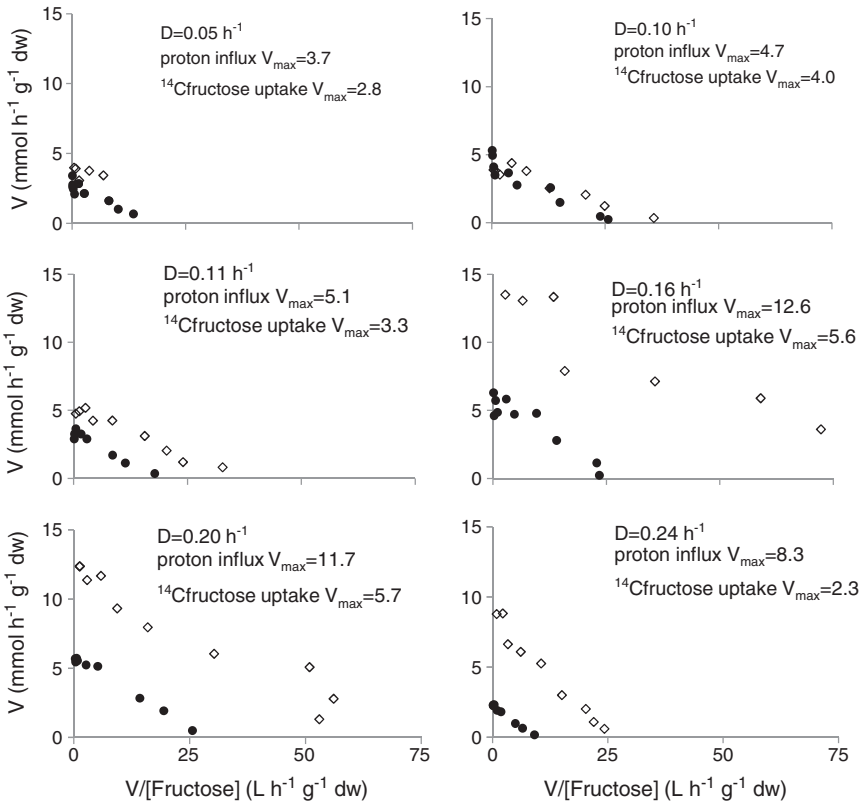

Fig. 4. Variable stoichiometry in chemostat cultures of strains *Saccharomyces cerevisiae* I4. Eadie–Hofstee plots of the initial uptake rates of D-[^{14}C]fructose (●) and proton symport (◊) by *S. cerevisiae* I4 during aerobic fructose-limited chemostat cultivation at various dilution rates.

turn imply that in normal physiological conditions, stoichiometries higher than 1:1 have hardly the opportunity to manifest themselves, suggesting that these stoichiometries may be a defective mode of operation that occurs when Fsy1 is artificially forced to operate at higher glycolytic fluxes. This is the case in high dilution rate aerobic chemostat cultures, where residual fructose concentration is insufficient to shut down the *FSY1* gene even when the glycolytic flux is high. The same holds for the strains expressing Fsy1 as sole hexose transporter (I3 and I4) because in these strains the *FSY1* gene is expressed constitutively [5]. In fact, the occurrence of this putative "malfunction" at higher glycolytic fluxes, underscores the need for stringent repression of the gene at high fructose concentrations, since at this range of concentrations the Fsy1 transporter is not only clearly dispensable because the Hxt proteins ensure efficient fructose transport, but may even be deleterious if it operates in an energetically particularly costly mode.

3.4. Conclusion

In summary, we found a variable fructose:H^+ stoichiometry for Fsy1 mediated transport and put forward the hypothesis that the energetically unfavorable 1:2 (or higher) stoichiometry may constitute a defective mode of operation that does not have a significant role under the conditions the yeast cells normally encounter. Our results are consistent with a fructose scavenging role for the Fsy1 transporter, meaning that it is used only when fructose is scarce. This implies that operation of Fsy1 normally occurs when the glycolytic flux is low, favoring a fructose:H^+ stoichiometry of 1:1, and metabolism is respiratory, minimizing the relative energetic burden of active fructose transport. Although higher stoichiometries may have little physiological significance as long as the *FSY1* gene is down regulated at high fructose concentrations, this mode of operation opens an interesting avenue for the improvement of ethanol yield from fructose rich substrates [32].

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2012.08.011.

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