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Role of de novo protein synthesis in the interconversion of glucose transport systems in the yeast *Pichia ohmeri*

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Glucose-repressed cells of the yeast *Pichia ohmeri* IGC 2879 transported glucose by facilitated diffusion. Derepression led to the formation of a glucose/proton symport and the simultaneous reduction of the facilitated diffusion capacity by about 70%. Cycloheximide prevented this interconversion indicating its dependence on de novo protein synthesis (proteosynthetic interconversion). In buffer with 2% glucose the glucose/proton symport suffered irreversible inactivation while the facilitated diffusion system was simultaneously restored. This reverse interconversion process did not require de novo protein synthesis as indicated by its lack of sensitivity to cycloheximide (degradative interconversion). Thus the glucose/proton symport system appeared to consist of about 70% of the facilitated diffusion proteins turned silent through association with additional protein(s) the latter being sensitive to glucose-induced repression and glucose-induced inactivation.

Introduction

Recently it was reported from our laboratory [1] that the yeast *Candida wickerhamii* had two interconvertible transport systems for glucose. Under repressed conditions a facilitated diffusion system was formed. Derepression allowed the formation of a glucose proton symport while the activity of the facilitated diffusion system could no longer be detected. When derepressed cells were suspended in buffer with glucose the latter reemerged while the proton symport was inactivated. Since no suitable inhibitor of protein synthesis in *C. wickerhamii* was available the fundamental question whether the interconversion processes required de novo protein synthesis remained an open one. A current survey of our yeast culture collection with respect to glucose transport modes in yeast led to the detection of a strain of *Pichia ohmeri* that displayed interconversion of glucose transport systems and was sensitive to cycloheximide. Here we report on the role of de novo protein synthesis in the interconversion processes in *P. ohmeri*.

Materials and Methods

Microorganism, growth and interconversion conditions. Pichia ohmeri IGC 2879 was originally obtained from the Northern Regional Research Laboratory, Peoria, IL under number NRRL Y-1922. It was maintained on glucose (2% w/v)/peptone (1% w/v)/yeast extract (0.5% w/v)/agar.

For growth under conditions of glucose repression a mineral medium with vitamins and 2% w/v

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glucose [2] was used at 25 °C with mechanical shaking. Derepression was induced by starving the cells during 3 h in the same medium without glucose. Inactivation of the glucose proton symport was induced by suspending derepressed cells in 100 mM Tris-citrate buffer (pH 5) at 25 °C in the presence of 2% w/v glucose.

De novo protein synthesis was inhibited by adding cycloheximide (from Sigma) to the interconversion media to a final concentration of 10 $mg \cdot l^{-1}$. Though cycloheximide at the concentration used had itself an inhibitory effect on glucose transport, the inhibition was reversible and completely removed by the washing procedures that preceeded measurements of glucose uptake rates.

Measurements of glucose uptake rates. Cells were harvested from the glucose medium in the exponential phase (repressed cells) or from the glucose-less medium after starvation (derepressed cells), centrifuged, washed twice with ice-cold distilled water and resuspended in ice-cold distilled water with a final concentration of 30–40 mg dry wt./ml.

From this cell suspension, 20 µl amounts were mixed in 10 ml conical centrifuge tubes with 20 µl 100 mM Tris-citrate buffer (pH 5). The tubes were incubated at 25 °C in a water-bath and the reaction was started by the addition of D-[1-^{3}H]glucose (about 1 µCi/µmol) at the desired concentration. At different intervals (up to 20 s), uptake was stopped by dilution with 5 ml ice-cold distilled water. The suspensions were filtered immediately through Whatman GF/C membranes. The filters were washed with 10 ml ice-cold water and counted in 10 ml scintillation fluid that contained 10% w/v naphthalene, 0.7 w/v 2,5-diphenyloxazole (PPO) and 0.03% w/v 1,4-bis-2(5-phenyloxazolyl) benzene (POPOP) in 1,4-dioxane. Radioactivity was measured in a Beckman LS 8100 liquid scintillation system.

In the case of the glucose proton symport initial glucose uptake rates were also estimated by measuring initial proton uptake rates. Reproducible results were obtained by the use of a Radiometer (Copenhagen) RTS 822 Recording Titration System connected to a Rea 160 Titrigaph module for pH-stat measurements. To the electrode vessel were added 6.9 ml distilled water and 1 ml yeast suspension (final concentration 3.5–4.0 mg dry wt./ml). The pH was kept constant at pH 5 with 0.25 mM HCl as titrant. The desired amount of glucose in 0.1 ml distilled water was added and the addition of the titrant by the pH-stat was followed in the recorder as a function of time. The slopes of the initial parts of the curves were used to calculate the initial rates of proton uptake (experiments were done in triplicate).

For estimating the proton-glucose stoicheiometry by comparing proton uptake rates with the rates of uptake of labelled glucose it was essential to use in both types of experiment cell suspensions with the same density.

Interconversion calculations. To calculate the relative capacity of facilitated diffusion and proton symport during interconversion an iterative procedure described earlier [3] was applied to the experimental data.

Measurement of 3-O-methylglucose accumulation. Preliminary experiments showed that 3-*O*-methylglucose is a non-metabolizable analogue of glucose for *P. ohmeri* that exclusively uses the transport systems for glucose. Furthermore it is not readily phosphorylated by yeast hexokinase [4]. Either repressed or derepressed cells (80 µl) were added to 80 µl 100 mM Tris-citrate buffer (pH 5) and incubated at 25 °C with magnetic stirring. The reaction was started by the addition of 20 µl 9 mM 3-*O*-methyl[1-^{3}H]glucose (Amersham), about 2000 µCi/µmol. At appropriate times, 10 µl samples were taken from the reaction mixture and filtered immediately through Whatman GF/C membranes. The filters were washed twice with 10 ml ice-cold water and the radioactivity was counted as indicated above. The harvesting, washing and testing of the repressed cells were done in the presence of 10 $mg \cdot l^{-1}$ cycloheximide. Otherwise the cells would form minute amounts of the accumulative glucose/proton symport during the time necessary for these operations.

Results

Characterization of the glucose transport systems of Pichia ohmeri

Cells grown in glucose medium (repressed cells) did not give significant proton signals when glucose was added to aqueous suspensions of the yeast. Lineweaver-Burk plots of the initial uptake

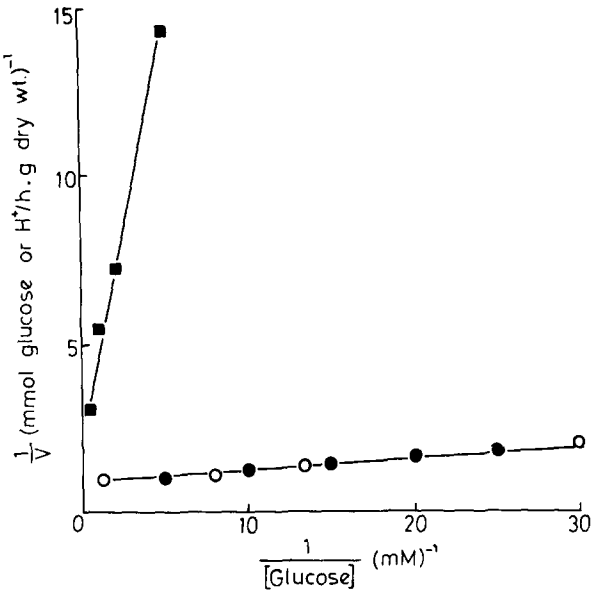

Fig. 1. Lineweaver-Burk plots of initial uptake rates of glucose by *Pichia ohmeri* IGC 2879. ■, Uptake of labelled glucose by glucose-grown cells; ●, uptake of labelled glucose by starved cells; ○, uptake of protons by starved cells after addition of glucose.

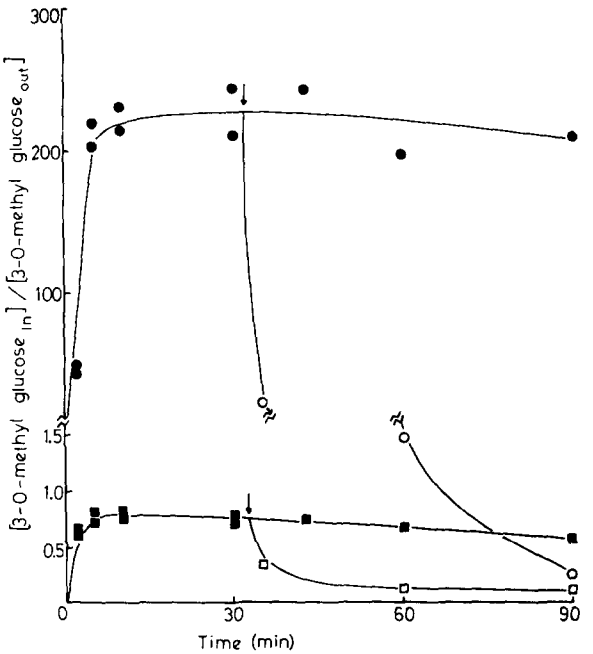

Fig. 2. Uptake of labelled 3-*O*-methylglucose by *Pichia ohmeri* IGC 2879. Initial extracellular 3-*O*-methylglucose concentration 1 mM. ●, Uptake of 3-*O*-methylglucose by starved cells; ■, uptake of 3-*O*-methylglucose by glucose-grown cells harvested and assayed in the presence of cycloheximide 10 $mg \cdot l^{-1}$. At the times indicated by the arrows, one half of each suspension received glucose to a final concentration of 250 mM. ○, Glucose-induced counterflow of 3-*O*-methylglucose in starved cells; □, glucose-induced counterflow of 3-*O*-methylglucose in glucose-grown cells.

rates of labelled glucose by such cells revealed that the yeast had an uptake system for glucose that displayed saturation kinetics with a capacity (V_{max}) of 0.5–1.5 mmol glucose/h per g dry wt. and a half-saturation constant (K_m) of 1–5 mM (Fig. 1). Transport of labelled 3-*O*-methylglucose was equilibrating and counterflow was induced by unlabelled glucose (Fig. 2). Based on these observations we concluded that the glucose transport system of repressed cells represented facilitated diffusion.

Proteosynthetic interconversion

Repressed cells were starved at 25 °C with shaking in glucose-less growth medium. At time zero the Eadie-Hofstee plot of the initial uptake rates of labelled glucose was linear with the K_m value characteristic for the facilitated diffusion system. During starvation the plots became biphasic displaying a low-affinity segment corresponding to facilitated diffusion and a high-affinity segment representing an emerging second system of glucose transport. The low-affinity segment decreased while the high-affinity segment increased with time. After 3 h of starvation there was no further change in the relative weights of the two transport systems and some residual facilitated diffusion capacity remained (Fig. 3).

The relative capacities (V_{max}) of the two systems during starvation were calculated by an iterative procedure [3]. The absolute value of the residual facilitated diffusion capacity varied from experiment to experiment; typically it was about 30% of its original capacity (Fig. 4).

Starvation in the presence of 10 $mg \cdot l^{-1}$ cycloheximide did not change significantly the capacity of facilitated diffusion and the high-affinity transport system did not emerge indicating the need of de novo protein synthesis in its formation (Fig. 3).

Characterization of high-affinity transport of glucose in Pichia ohmeri

Proton signals were observed when glucose was added to aqueous suspensions of cells that had been starved during 3 h in glucose-less growth medium (derepressed cells). At low glucose concentrations at which the contribution of residual facilitated diffusion to the total uptake rate was not significant, the Lineweaver-Burk plots of initial proton uptake rates as well as of the initial uptake rates of labelled glucose were coincident (Fig. 1), which revealed that one proton was taken up for each glucose molecule transported. The affinity of the derepressed transport system for glucose ($K_m = 50–150$ μM) was 10–20-fold that of the facilitated diffusion system while the capacity was similar ($V_{max} = 1–1.5$ mmol glucose/h per g dry wt.). Transport of labelled 3-*O*-methylglucose was strongly accumulative (over 220-fold) and unlabelled glucose induced efflux (Fig. 2). The protonophore CCCP (1 mM) added one minute before starting the reaction prevented accumulation (not shown). These observations led us to conclude that the high-affinity transport system for glucose that emerged during starvation was a glucose-proton symport.

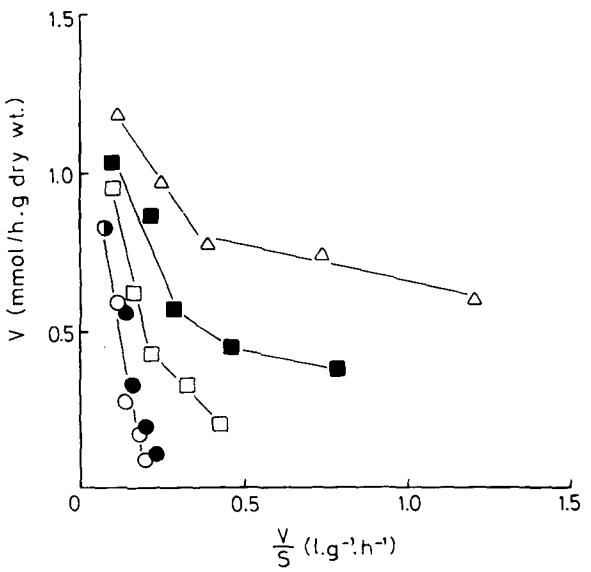

Fig. 3. Eadie-Hofstee plots of the initial uptake rates of labelled glucose by washed glucose-grown cells of *Pichia ohmeri* IGC 2879 incubated in glucose-less growth medium during 0 h (○), 0.5 h (□), 1.5 h (■); 3 h (△) and during 3 h (●) in the presence of cycloheximide 10 mg·l^{-1}.

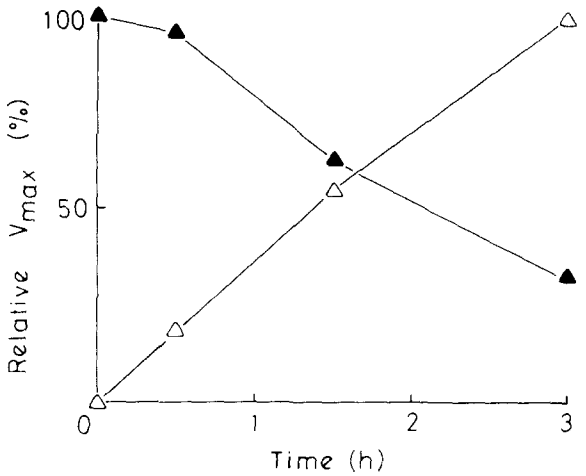

Fig. 4. Variation of the maximum velocities of the facilitated diffusion system for glucose transport (▲) and the glucose-proton symport (△) of *Pichia ohmeri* IGC 2879 during carbon starvation of glucose-grown cells calculated by an iterative procedure [3] from the data shown in Fig. 3.

Degradative interconversion

When derepressed cells were incubated at 25 °C with shaking in buffer with 2% glucose, the proton symport function gradually decreased while the capacity of facilitated diffusion increased with time. After 8 h of incubation facilitated diffusion was the only system present (Fig. 5). Iterative calculations showed that the latter's capacity increased about 2-fold during the process while about 40% of the original capacity present at time zero of the starvation experiment was irretrievably lost. Cycloheximide at 10 mg·l^{-1} did not affect this reverse interconversion process indicating that the restoration of the facilitated diffusion system did not require protein synthesis and was apparently dependent only on the glucose-induced inactivation of the glucose-proton symport function.

When the cells were again incubated in glucose-less growth medium some glucose-proton symport activity reappeared (not shown). Cycloheximide (10 mg·l^{-1}) prevented the reappearance indicating that de novo protein synthesis rather than reactivation had taken place. We concluded that the glucose-induced inactivation of the glucose-proton symport was irreversible and led to the restoration of the function of silent facilitated diffusion proteins.

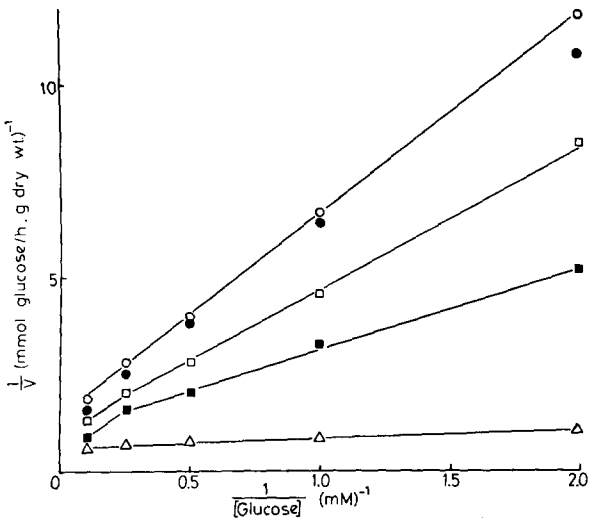

Fig. 5. Lineweaver-Burk plots of the initial uptake rates of labelled glucose by starved cells of *Pichia ohmeri* IGC 2879 in 100 mM Tris-citrate buffer (pH 5) with 2% (w/v) glucose during 0 h (△), 2 h (■), 6 h (□), 8 h (○) and 8 h (●) in the presence of cycloheximide 10 mg·l^{-1}.

Discussion

The results obtained earlier with *C. wickerhamii* [1] together with the data presented here on the role of de novo protein synthesis in the interconversion of glucose transport systems in *P. ohmeri* suggest the following mechanisms. When these yeasts are growing under conditions of glucose repression only the facilitated diffusion system is formed and is operative. Indeed it makes physiological sense that a high-affinity, energy-dependent proton symport is not formed when the glucose concentration is high enough to ensure rapid facilitated diffusion. The facilitated diffusion system is subject neither to glucose-induced repression nor to glucose-induced inactivation. Under derepressed conditions (an) additional protein(s) is (are) formed which associate(s) with the facilitated diffusion system thus forming a glucose proton symport while the facilitated diffusion function becomes dormant (proteosynthetic interconversion).

The amount of facilitated diffusion protein that is used up (and silenced) in the assembly of the glucose proton symport system would depend on the latter's final capacity. In *P. ohmeri* the maximum amount of additional transport protein the cells were able to synthesize during starvation left about 30% of the facilitated diffusion capacity in a functioning state.

The protein(s) that confer(s) proton symport properties to the system is (are) subject to glucose-induced repression and to glucose-induced inactivation. Thus, when conditions of glucose repression are reestablished, the latter protein(s) suffer(s) inactivation, the proton symport function is lost and the facilitated diffusion function is restored (degradative interconversion). It was tempting to propose the name 'proteolytic' interconversion for the cycloheximide-insensitive part of the interconversion process, as a counterpart to the name 'proteosynthetic' interconversion proposed for the cycloheximide-sensitive part. However, no experimental evidence is available indicating that degradative interconversion is due to proteolytic action on the proton glucose symport protein(s). Furthermore, though we found that the glucose-induced inactivation of the proton-glucose symport was irreversible after completion of the interconversion process, it cannot be excluded that the inactivation might be reversible during the early part of the process as has been found to be the case in the glucose-induced inactivation of the fructose-1,6-diphosphatase of *Saccharomyces cerevisiae* [5–8].

The occurrence of multiple transport systems for sugars and other substrates in a wide variety of cell types has been reported on numerous occasions. It remains to be seen whether interconversion mechanisms of the type described above and earlier [1] are also of widespread occurrence. Published evidence suggests that similar mechanisms may be operative in the regulation of hexose transport systems in *Saccharomyces cerevisiae* [9,10] and in *Neurospora crassa* [11], as well as of transport of phosphate in *Candida tropicalis* [12] and of potassium ions in *S. cerevisiae* [13].

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