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Catabolite interconversion of glucose transport systems in the yeast *Candida wickerhamii*

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Candida wickerhamii **IGC 3244 growing in glucose medium transported glucose by facilitated diffusion (at 25°C and pH 5, the K_s value was 1.7 mM and the V_{max} value was 1.6 mmol/h per g dry wt.), while cells grown under derepressed conditions produced a glucose proton symport (at 25°C and pH 5, the K_s value was 0.18 mM and the V_{max} value was 1.8–1.9 mmol/h per g dry wt.). In each case, the Lineweaver-Burk plot of initial uptake rates was linear, indicating the presence of a single system. In buffer with 2% glucose, the symport suffered catabolite inactivation while the facilitated diffusion system emerged concomitantly in such a way that the combined V_{max} remained nearly constant. During the conversion process, the Linewaver-Burk plots were biphasic, indicating the transitory co-existence of the two systems. A model is proposed that envisions the proton symport as composed of the facilitated diffusion system in association with (an)other transport protein(s), the latter being sensitive to carbon catabolite repression and inactivation.**

Introduction

The yeast *Candida wickerhamii* is able to ferment cellobiose and has been proposed as an auxiliary agent for the bioconversion of cellulose into ethanol [1–3]. It produces a β-glucosidase which hydrolyses cellobiose mainly on the cell surface [2,3]. We found that the glucose thus produced is transported into the yeast cell by a proton symport. Under conditions of glucose repression, the symport is not formed and glucose is transported by facilitated diffusion. Here, we present data indicating that catabolite inactivation induced by glucose converts the proton symport into the facilitated diffusion system.

Materials and Methods

Microorganism and growth conditions. C. wickerhamii IGC 3244 was originally obtained from the Yeast Division of the Centraalbureau voor Schimmelcultures, Delft, The Netherlands under number CBS 2928. 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 catabolite repression, a mineral medium with vitamins and 0.5% w/v glucose [4] was used at 25°C with mechanical shaking. Derepressed conditions were obtained by substituting 0.5% w/v cellobiose for glucose in the above medium.

Measurement of glucose uptake rates. Cells were harvested in early exponential phase (A_{640} of at least 0.25, measured in a Bausch&Lomb Spectronic 21), centrifuged, washed twice with ice-cold distilled water and resuspended in ice-cold dis-

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Abbreviation: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

tilled water with a final concentration of about 25 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-scintillator counter.

In the case of the glucose proton symport glucose uptake rates were also estimated by measuring proton uptake with a standard pH meter PHM 62 (Radiometer, Copenhagen) connected to a flatbed Perkin-Elmer 024 Recorder. The pH electrode was immersed in a water-jacketed chamber of 10 ml capacity kept at 25°C and provided with magnetic stirring. To the chamber were added 2.9 ml distilled water and 1 ml yeast suspension. The pH was adjusted to 5 and a base line was obtained. The desired amount of glucose in 0.1 ml was added and the subsequent alkalinization was followed in the recorder. The slope of the initial part of the pH trace was used to calculate the initial uptake rate. Calibration was performed with HCl and NaOH.

Measurement of L-*sorbose accumulation.* Preliminary experiments showed that L-sorbose is a non-metabolizable analogue of glucose for *C. wickerhamii* that exclusively uses the transport systems for glucose. Either repressed or derepressed cells (80 μl, 25 mg dry wt./ml water) were added to 80 μl 100 mM Tris-citrate buffer (pH 5) and also to 80 μl buffer containing carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and incubated at 25°C with magnetic stirring. The reaction was started by the addition of 20 μl 10 mM L-[U-^{14}C]sorbose (about 3000 cpm/nmol). At appropriate times, 10-μl samples were taken from the reaction mixture and filtered immediately through Whatman GF/C membranes. The filters were washed three times with 10 ml ice-cold water and the radioactivity was counted as indicated above.

The intracellular concentration of sorbose was calculated assuming that 1 mg dry wt. of the yeast contained 2.0 μl of intracellular water [5].

Estimation of residual protein synthesis. Residual protein synthesis was measured by the incorporation of L-[U-^{14}C]leucine (Amersham) into cold trichloroacetic acid-insoluble material. It was established in preliminary experiments that cells grown under derepressed conditions as described above were able to transport leucine. Isotope (10 μl) was added to 40 μl of the suspension containing about 4 mg cells and 2% w/v glucose in 100 mM Tris-citrate buffer (pH 5) to a concentration of 10 μCi/ml. After different intervals (up to 3 h) of incubation at 25°C with magnetic stirring, the reaction was stopped in each tube with 1 ml ice-cold 10% trichloroacetic acid and 1 ml 10% w/v lauryl sulphate. Samples were then incubated for 30 min at 90°C. The acid-insoluble material was collected by filtration on Whatman glass-fiber filters, washed three times with 5 ml ice-cold 5% trichloroacetic acid, twice with a mixture of diethyl ether/ethanol (1 : 1) and counted.

Results

Cells grown in glucose medium did not give significant proton signals when glucose was added to aqueous suspensions of the yeast. Lineweaver-Burk plots of the initial rates of uptake of labeled glucose by such cells revealed that the yeast had an uptake system for glucose that displayed Michaelis-Menten kinetics with a capacity (V_{max}) of 1.6 mmol glucose/h per g dry wt. and a half-saturation constant (K_s) of 1.7 mM (Fig. 1). Furthermore, transport of labeled L-sorbose, a non-metabolizable analogue of glucose, was equilibrating and counterflow was induced by glucose (Fig. 2). Based on these observations, it was concluded that the glucose-transport system of glucose-grown cells represented facilitated diffusion.

Proton signals were observed when glucose was added to aqueous suspensions of cells that had been grown in batch culture with cellobiose as the carbon source. Lineweaver-Burk plots of initial

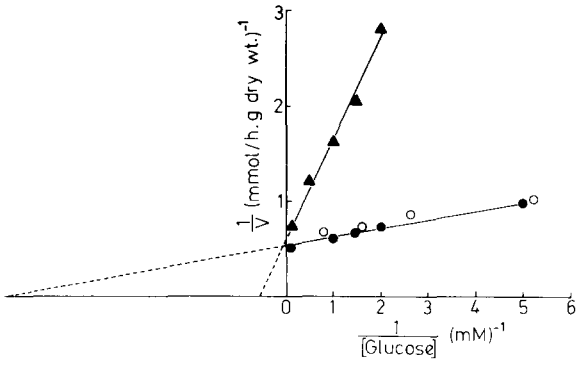

Fig. 1. Lineweaver-Burk plots of initial uptake rates of glucose by *C. wickerhamii* IGC 3244. ▲, Uptake of labeled glucose by glucose-grown cells; ●, uptake of labeled glucose by cellobiose-grown cells; ○, uptake of protons after addition of glucose by cellobiose-grown cells.

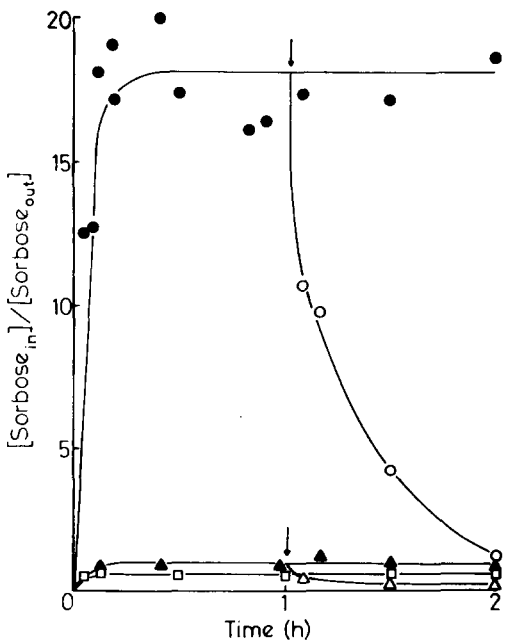

Fig. 2. Uptake of labeled L-sorbose by *C. wickerhamii* IGC 3244. Initial extracellular sorbose concentration 1.1 mM. ●, Uptake of sorbose by cellobiose-grown cells; □, uptake of sorbose by cellobiose-grown cells in the presence of 1 mM CCCP added 1 min before the reaction was started; ▲, uptake of sorbose by glucose-grown cells. 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 sorbose in cellobiose-grown cells; △, glucose-induced counterflow of sorbose in glucose-grown cells.

glucose uptake rates calculated from the slopes of the proton signals of cellobiose-grown cells as well as from the initial uptake rates of labeled glucose were coincident (Fig. 1) which revealed that one proton was taken up for each glucose transported. The affinity of the system for glucose was about 10-fold that of the facilitated diffusion system ($K_s = 0.18$ mM) while the capacity was similar ($V_{max} = 1.8–1.9$ mmol glucose/h per g dry wt.). Furthermore, transport of labeled L-sorbose was accumulative, the accumulation was prevented by the uncoupler CCCP, while glucose induced counterflow (Fig. 2). These observations, together with the association of glucose uptake with proton influx in a 1 : 1 ratio, led us to conclude that the glucose-transport system of cellobiose-grown cells was a proton symport.

The Lineweaver-Burk plots (Fig. 1) of glucose transport in the repressed and the derepressed cells did not display breaks which indicated that in either case only one transport system was measurably operating.

Derepressed cells grown in cellobiose medium were suspended in 100 mM Tris-citrate buffer (pH 5) and incubated at 25°C in the presence of 2% w/v glucose and in its absence. Samples were taken at intervals and the activity of the proton symport was avaliated by measuring initial proton uptake rates at saturating concentrations of glucose (6 mM). In the control experiment, the activity was still over 60% of its initial value after 5 h of incubation. In the presence of glucose, the activity decreased rapidly with time, was less than 10% of the original activity at 2.5 h and was hardly measurably thereafter with the pH electrode. Fig. 3 shows the results of a typical experiment.

While glucose transport by the proton symport calculated from proton signals decreased with time in the presence of glucose, the effect of glucose on its transport systems as measured with labeled glucose was more complex. Fig. 4 shows Lineweaver-Burk and Eadie-Hofstee plots of the initial transport rates measured with labeled glucose in derepressed cells incubated in buffer with 2% glucose. While there was no significant effect on the total capacity (V_{max}) of glucose uptake, the proton symport system was gradually substituted by facilitated diffusion. At time zero, the plots were linear with K_s values characteristic for the sym-

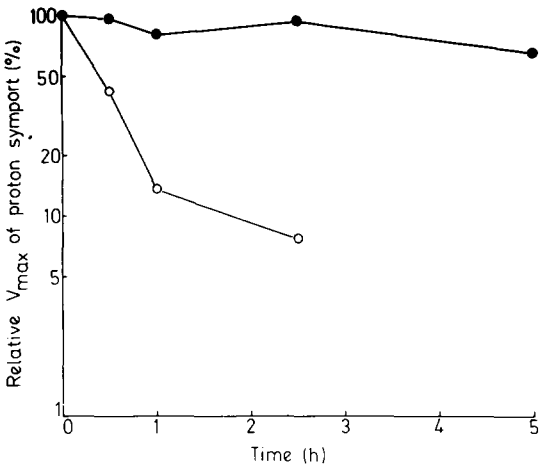

Fig. 3. Relative rates of the initial proton uptake after addition of glucose by washed samples taken from suspensions in 100 mM Tris-citrate buffer (pH 5) of cellobiose-grown cells of *C. wickerhamii* IGC 3244. ○, Activity of cells preincubated in buffer with 2% w/v glucose; ●, activity of cells preincubated in buffer without glucose.

port. After 5 h, the plots were again linear with the much higher K_s values characteristic for facilitated diffusion. At intermediate times, the plots displayed high-affinity and low-affinity segments, the latter increasing and the former decreasing with time.

The inactivation of the proton symport by glucose, whatever the molecular mechanism, represents catabolite inactivation by definition [6,7]. The concomitant emergence of facilitated diffusion raised the question whether de novo protein synthesis or a conversion process was the underlying mechanism. Inactivation experiments in the presence of the following inhibitors of eucaryotic protein synthesis, cycloheximide, ethionine and puromycin, were not conclusive since the yeast was resistant to the former while the latter two interfered with membrane transport and did not reduce the time of residual protein synthesis. By the use of labeled leucine it was found however that under the conditions of the inactivation experiments residual protein synthesis was completed within about 30 min (Fig. 5) while the catabolite inactivation of the proton symport and the concomitant emergence of facilitated diffusion was only completed after more than 2.5 h (Fig. 4).

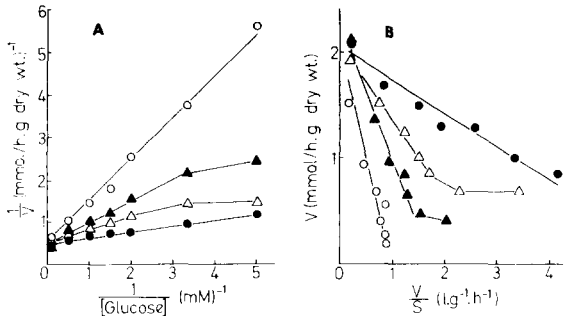

fig. 4. Lineweaver-Burk plots (A) and Eadie-Hofstee plots (B) of the initial uptake rates of labeled glucose by washed cellobiose-grown cells of *C. wickerhamii* IGC 3244 after preincubation in 100 mM Tris-citrate buffer (pH 5) with 2% w/v glucose during 0 h (●), 1 h (△), 2.5 h (▲) and 5 h (○).

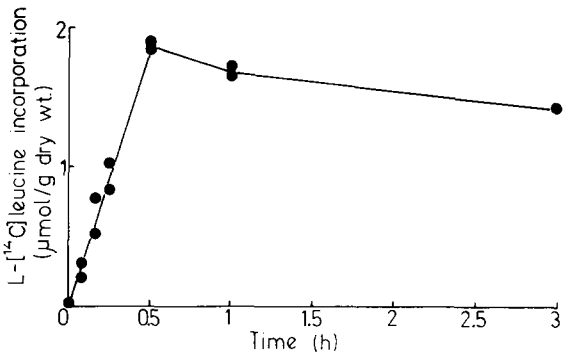

Fig. 5. Residual protein synthesis measured as L-[U-^{14}C]leucine incorporation into acid-insoluble cell material by cellobiose-grown cells of *C. wickerhamii* as used in the inactivation experiments depicted in Figs. 3 and 4.

We concluded that the appearance of the facilitated diffusion system during catabolite inactivation of the symport was probably not due to de novo protein synthesis but represented the conversion of one system into the other. Catabolite interconversion appears to be a suitable name for the process.

Discussion

Van den Broek and Van Steveninck [8] have shown that proton symport and facilitated diffusion using the same transporter yield linear Lineweaver-Burk plots of the initial uptake rates. In such a system, the steady-state concentration of

each form of the carrier at either side of the membrane is interlinked with those of all other forms through rate constants, equilibrium constants and substrate concentrations. The linear kinetics is a consequence of this interlinkage.

In *C. wickerhamii,* facilitated diffusion of glucose occurred in repressed cells, a glucose proton symport in derepressed ones. In either case, the Lineweaver-Burk plots were linear. During catabolite inactivation of the proton symport, facilitated diffusion emerged concomitantly and the Lineweaver-Burk and Eadie-Hofstee plots were biphasic, indicating the transitory coexistence of the two transport modes. The nonlinearity of the plots indicated that proton symport and facilitated diffusion of glucose in *C. wickerhamii* use distinct transport systems.

During the conversion process, the sum of the maximum velocities of the two transport systems was not noticeably changed. Since the V_{max} values of the two systems at the beginning and at the end of the conversion process, respectively, were similar, this constancy indicates a one for one exchange of the carrier molecules. Such an ordered conversion process suggests in turn the existence of a structural linkage between the two systems. The nonlinearity of the Lineweaver-Burk and Eadie-Hofstee plots during the conversion process implies that this linkage is not governed by reversible binding and diffusional events. We propose the following model.

Under growth conditions of carbon catabolite repression, *C. wickerhamii* produces a system for the facilitated diffusion of glucose. When the cells are growing under derepressed conditions, one or more additional transport proteins are synthesized which combine with the facilitated diffusion system to form the proton symport. When repressing conditions are reestablished, the additional transport protein(s) suffer(s) catabolite inactivation and are(is) removed from the carrier. As a consequence, during catabolite inactivation the proton symport is gradually reduced to the underlying facilitated diffusion system. A similar model was discussed earlier by Matern and Holzer [9] to explain glucose effects on the galactose transport systems of *Saccharomyces cerevisiae.*

The model implies that the carbon catabolite conversion of the two systems for glucose takes place without de novo protein synthesis. Though we found that the conversion process proceeded long after the net incorporation of added leucine had stopped, it cannot be concluded that no de novo protein synthesis occurred at all during the later stages of the process. In the hope of clarifying this matter, we are now surveying our yeast culture collection in search for a strain that displays catabolite interconversion of glucose-transport systems and is sensitive to cycloheximide.

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