Regulation of Glucose Transport in *Candida utilis*

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The transport systems for glucose present in *Candida utilis* cells, growing in batch and continuous cultures on several carbon sources, have been studied. Two different systems were found: a proton symport and a facilitated diffusion system. The high-affinity symport (K_m for glucose about 15 μ M) transported one proton per mole of glucose and was partially constitutive, appearing in cells grown on gluconeogenic substrates such as lactate, ethanol and glycerol. It was also induced by glucose concentrations up to 0.7 mM and repressed by higher ones. The level of repression depended on the external glucose concentration at which cells had grown in a way similar to that shown by the maltose-uptake system, so both systems seem to be under a common glucose control. Initial uptake by facilitated diffusion, the only transport system present in cells growing at glucose concentrations higher than 10 mM, showed a complex kinetic dependence on the extracellular glucose concentration. This could be explained either by the presence of at least two different systems simultaneously active, one with a K_m around 2 mM and the other with a K_m of about 1 M, or by the allosteric or hysteretic behaviour of a single carrier whose apparent K_m would oscillate between 2 and 70 mM.

INTRODUCTION

Several systems have been described for glucose transport in yeasts. In *Saccharomyces cerevisiae* a facilitated diffusion was reported many years ago (for a review see Eddy, 1982), and the first glucose- H^+ symport was described in *Rhodotorula gracilis* by Hofer & Misra (1978). In recent years both types of system have been found in some other yeasts (Spencer-Martins & van Uden, 1985; Loureiro-Días, 1987). For *Candida utilis*, a repressible H^+ symport was found in cells grown on lactate or under glucose limitation in the chemostat (Eddy & Hopkins, 1985). Postma *et al.* (1987) reported that glucose was transported with different affinities by cells grown at different growth rates in a glucose-limited chemostat.

In the present work we have studied the nature and regulation of the transport systems for glucose that appear in *C. utilis* cells grown under different conditions.

METHODS

Organism and culture conditions. The organism used was *Candida utilis* IGC3092 (CBS 890). It was maintained in slant cultures with YPG agar (yeast extract 5 g l⁻¹; peptone 1 g l⁻¹; glucose 2 g l⁻¹ and agar 2 g l⁻¹), and was grown in the synthetic medium described by van Uden (1967), with sugars as indicated in the text.

For continuous culture experiments, we used a Bioflo C30 fermenter (New Brunswick, NJ, USA) with a working volume of 750 ml. The temperature was 28 °C; the pH was maintained at 4.0 by the addition of 1 M-KOH using a pHstat device (Radiometer). The culture was carbon-limited at all the dilution rates studied, as shown by the constant cell yield obtained: 0.55 $\pm$ 0.042 g biomass per g total carbohydrate consumed (mean $\pm$ SD; seven

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determinations). Steady-state cells were harvested by centrifugation in an Eppendorf centrifuge for 10 s and washed twice with ice-cold demineralized water.

For batch cultures we used 2-litre shake flasks with 500 ml medium in an orbital incubator at 28 °C. Cells were harvested in the mid-exponential phase (OD_{640} about 0.7) in a B20 International centrifuge at 4 °C and washed twice with ice-cold demineralized water.

Final suspensions of cells, from continuous or batch cultures, were prepared in demineralized water at a concentration of 50 mg dry weight ml^{-1} , kept on ice and used for transport measurements within 2 h.

Calculation of the specific consumption rate of glucose, q_G . The q_G value for chemostat-grown cells at each steady-state was calculated using the equation $q_G = D(S_0 - S)/X$, where D is the dilution rate, S_0 and S are the concentrations of glucose in the fresh medium and in the fermenter respectively, and X is the biomass concentration in the fermenter.

Measurement of the initial rate of glucose transport using [$1-^3H$]glucose. We used the method described earlier (Loureiro-Dias & Peinado, 1984), with some modifications. We suspended 1 mg (dry weight) of cells in 40 μl 50 mM-Tris/citrate buffer in a conical tube incubated in a water bath at 28 °C. After 2 min incubation, 10 μl of a solution of the labelled sugar (specific activity about 10 GBq mol^{-1}) were added. Transport was stopped 10 s later by the addition of 5 ml ice-cold water. Cells were filtered under vacuum through Whatman GF/C membranes, washed with 5 ml ice-cold water, and radioactivity was measured in a liquid scintillation system.

To study the kinetics of the high-affinity system we used only 0.1 mg of cells and a specific activity ten times higher; otherwise, due to the low glucose concentrations used, the sugar was significantly consumed during the assay. In some experiments we measured the capacity of this system as the initial rate of transport for a concentration of 0.1 mM-glucose, since at this concentration the high-affinity system was saturated and the transport through the low-affinity one(s) was not significant. To study the kinetics of the low-affinity facilitated diffusion, we varied the concentration of glucose between 0.125 and 500 mM. This was accomplished by adding a fixed amount of labelled sugar (0.1 MBq) to all the assays and varying the specific activity with concentration. In all cases we checked that 10 s was within the time interval during which the uptake of radioactive glucose was linear with time.

Measurement of the initial rate of glucose transport by H^+ uptake. We used the method previously described (Loureiro-Dias & Peinado, 1984) with suitable concentrations of glucose.

Measurement of accumulation ratios. Accumulation ratios were calculated for L-[$U-^{14}C$]sorbitose, as already described (Loureiro-Dias, 1987). In previous experiments, mutual competitive inhibition of transport of L-sorbitose and glucose was observed in this strain.

Maltose consumption capacity. Maltose consumption capacity in chemostat-grown cells was evaluated by the ability of cell suspensions to acidify the unbuffered external environment upon addition of maltose (10 mM initial concentration). The same conditions were used as those utilized to measure initial H^+ uptake. Relative values were calculated taking as 100% the value obtained with cells grown in the chemostat at the lowest dilution rate (derepressed cells).

Determination of sugars. Glucose was determined by the oxidase-peroxidase method (Boehringer Mannheim). Total sugars (glucose plus maltose) were determined by the phenol/sulphuric acid method (Herbert *et al.*, 1971). Maltose was taken as the difference between the two values.

RESULTS

Kinetics of growth and glucose consumption in chemostat-grown cells

Candida utilis IGC3092 was grown in the chemostat on a mixture of glucose (3 g l^{-1}) and maltose (1 g l^{-1}), at several dilution rates, as described in Methods. The steady-state values of biomass and sugar concentrations at each dilution rate were determined (Fig. 1). Maltose consumption was used as an indicator of glucose repression, in an attempt to check if the same repression mechanisms acting on maltose consumption would act on some of the transport systems for glucose. We had previously shown (Peinado *et al.*, 1987) that glucose represses maltose consumption in the chemostat, so the maltose consumption capacity of the cells was determined to verify the conditions at which glucose repression was triggered. Fig. 1 shows that this happened at a dilution rate of 0.25 h^{-1} , corresponding to a glucose concentration of 0.7 mM. Increasing the dilution rate led to decrease in maltose consumption capacity and, correspondingly, the maltose concentration in the fermenter increased. When the concentration of glucose reached 9.6 mM, repression was complete and the maltose concentration in the fermenter was that of the fresh medium.

When the kinetics of growth was studied, we found that the dependence of specific growth rate on the external glucose concentration did not follow the Monod equation. Instead, a non-

Fig. 1

Fig. 1. Steady-state concentrations of glucose (■) and maltose (□) in the chemostat, and relative maltose consumption capacity (Δ) of *C. utilis* at several dilution rates (D).

Fig. 2

Fig. 2. Eadie-Hofstee plot of the specific glucose consumption rate, q_G , of *C. utilis* and the steady-state glucose concentration in the chemostat.

linear Eadie-Hofstee plot was obtained. From this non-linear plot (not shown) a maximum specific growth rate of 0.47 h^{-1} was extrapolated from the higher D values and apparent affinities for glucose (K_s) of about 0.83 and 0.03 mM were calculated from the higher and lower D values respectively. As this was a carbon-limited culture, similar changes in the apparent affinity for glucose of the specific rate of glucose consumption were to be expected, and they were indeed observed. Fig. 2 shows an Eadie-Hofstee plot of the specific consumption rate of glucose versus the corresponding glucose concentration in the fermenter. From these data, a maximum rate of glucose consumption of $4.9 \text{ mmol g}^{-1} \text{ h}^{-1}$ and apparent K_m values of about 1.1 and 0.03 mM were calculated. These results showed that the apparent affinity for glucose uptake depended on the concentration of the sugars at which the cells were growing. The changes in apparent affinity could correspond to changes in the transport system utilized by the yeast, so we tried to characterize and identify these potentially different systems.

Simultaneous presence of a H^+ symport and a facilitated diffusion system for glucose transport in chemostat grown cells.

The initial transport rate of glucose was assayed in washed cells that had grown in the chemostat at a dilution rate of 0.2 h^{-1} with a glucose concentration of 0.3 mM .

When transport was measured with radioactive glucose (see Methods), the Eadie-Hofstee plot of the initial velocities against the concentration of glucose was biphasic (Fig. 3), indicating the presence of at least two transport modes with affinities around 20 mM and $20 \mu\text{M}$. To determine whether the high-affinity form was a H^+ symport that had been previously described for *C. utilis* (Eddy & Hopkins, 1985), the initial transport rate was measured by following H^+ uptake by a cell suspension after glucose addition, as described in Methods. The Eadie-Hofstee plot of H^+ uptake, also shown in Fig. 3, was linear, showing an affinity and capacity similar to those of the high-affinity component detected previously by the radioactive glucose method. We concluded that the high-affinity form was a H^+ symport with a stoichiometry of one mole of glucose per proton cotransported, as Eddy & Hopkins (1985) had found for cells grown with lactate or low concentrations of glucose. This symport could be used also by sorbose, as competition experiments showed.

To distinguish further between the two forms of transport, their ability to accumulate sorbose was investigated. The symport was studied in cells grown in the chemostat at low dilution rate, and the low-affinity system in cells grown in batch culture with 2% (w/v) glucose (in these batch-

Fig. 3

Fig. 3. Eadie-Hofstee plot of the initial rate of glucose transport by cells of *C. utilis* previously grown in the chemostat at a dilution rate of 0.2 h^{-1} . The transport was measured by following $[^3\text{H}]$ glucose incorporation ($\bullet$) or H^+ uptake ($\circ$).

Fig. 4

Fig. 4. Accumulation of L-[U- ^{14}C]sorbitose by *C. utilis* cells grown under derepression conditions (low dilution rate in the chemostat) ($\blacksquare$, $\square$) and repression conditions (2%, w/v, glucose batch culture) ($\blacktriangle$, $\triangle$). Open symbols follow the addition of 1 mM-glucose.

grown cells no H^+ uptake was observed upon addition of glucose). As expected, the symport accumulated the non-metabolizable analogue up to 15-fold, and addition of glucose produced counterflow (Fig. 4). In contrast, the low-affinity transport system of batch-grown cells was equilibrative: i.e. it was a facilitated diffusion.

These experiments showed that the cells could display two clearly distinct transport systems: a H^+ symport and a facilitated diffusion. Apparently, both systems could be present simultaneously in chemostat-grown cells (see Fig. 3). We will henceforth refer to the high-affinity system as the H^+ symport and to the low-affinity system as the facilitated diffusion.

Regulation of the glucose- H^+ symport

The glucose- H^+ symport was also found in cells grown in batch culture on lactate, as described by Eddy & Hopkins (1985), and on other gluconeogenic carbon sources such as ethanol and glycerol, but not on glucose. The results, summarized in Table 1, showed that the symport was partially constitutive and repressed by glucose. Moreover, the symport could be found in cells previously grown under repressing conditions (2% glucose), after 4 h starvation in buffer. This process was inhibited by the presence of cycloheximide in the starvation buffer, indicating that derepression required *de novo* protein synthesis (Table 1). When cells grown in the chemostat at different growth rates were analysed for their capacity to transport glucose with high affinity, measuring both the incorporation of radioactive glucose and the uptake of H^+ , the results confirmed the stoichiometry 1:1 (1.08 ± 0.27 , $n = 8$), and also showed that the symport was induced by glucose. As shown in Fig. 5, cells grown in the chemostat at the lowest glucose concentration studied had a symport capacity similar to the basal level detected in ethanol-grown cells (see Table 1). When the cells were grown at higher glucose concentrations their symport capacity increased, reaching a value three times the basal level at a glucose concentration of 0.7 mM , which corresponded to a dilution rate of 0.25 h^{-1} . Beyond this dilution rate, i.e. at higher glucose concentrations, repression was triggered, and at a glucose concentration of about 10 mM repression was complete, as happened with the maltose consumption capacity (see Fig. 1). Comparing Fig. 5 and Fig. 1 it can be concluded that both systems, the glucose- H^+ symport and the maltose consumption capacity, show the same glucose dependence in their repression, indicating a common underlying control mechanism.

Fig. 5. Glucose- H^+ symport capacity of *C. utilis* cells grown at several dilution rates (D) in the chemostat. The transport capacity was measured either by following H^+ uptake upon the addition of 10 mM-glucose (○), or with 0.1 mM- $[^3H]$ glucose (●).

Table 1. Uptake of glucose by the high-affinity system (H^+ symport) in *C. utilis* cells grown in batch culture on different substrates

$[1-^3H]$ Glucose incorporation was measured after 10 s incubation with saturating (0.1 mM) glucose. Results are mean values of at least two experiments.

Growth substrate	Uptake (mmol g ⁻¹ h ⁻¹)
Lactate	0.58
Ethanol	0.40
Glycerol	0.20
Glucose (2%, w/v)	
Exponential-phase cells	0.07
Exponential-phase cells after 4 h starvation	
Without cycloheximide	0.30
With cycloheximide	0.04

Features of the facilitated diffusion for glucose

Even the cells that had grown in the chemostat at the lowest glucose concentration (below 10 μ M), showed a biphasic Eadie-Hofstee plot, with a low-affinity component, as that of Fig. 3, when assayed for their initial transport capacity with glucose concentrations up to 100 mM. This means that the facilitated diffusion system was present. Although the capacity could not be determined precisely from these plots, the values obtained for the set of dilution rates studied (those of Fig. 5) were of the same order as the maximum rate of glucose uptake by batch-grown cells: about 5 mmol g⁻¹ h⁻¹. We concluded that the synthesis of the facilitated diffusion system does not seem to depend on the concentration of glucose.

The kinetics of the facilitated diffusion system was studied using mainly batch grown cells, in which only this system was present. Eadie-Hofstee plots of the initial velocities using glucose concentration up to 0.5 M were biphasic. Fig. 6 shows the results of a typical experiment. This non-linear behaviour could be explained by two different mechanisms: either there are two distinct permeases able to transport glucose by facilitated diffusion or there is a single permease whose affinity depends on the glucose concentration. In the case of the first possibility, K_m values of 2 mM and 1 M could be calculated for each permease, separating the values for initial glucose transport through each system from the experimental results, by iterative procedures. The second possibility could be further explored theoretically. Extrapolating the maximum velocity from Eadie-Hofstee plots such as that of Fig. 6, a Hill plot was prepared. Fig. 7 shows such a plot for the same data as those of Fig. 6. The system seemed to behave as a single permease that could be in two different states: at low glucose concentrations (below 7 mM) all the

Fig. 6

Fig. 7

Fig. 6. Eadie-Hofstee plot of the initial rate of transport of $[^3\text{H}]$ glucose by *C. utilis* cells grown in batch culture with 2% (w/v) glucose.

Fig. 7. Hill plot of the initial rate of transport of $[^3\text{H}]$ glucose by *C. utilis* cells grown in batch culture with 2% (w/v) glucose. The data are the same as those of Fig. 6; v_{max} was extrapolated from the upper part of that curve.

Table 2. Hill coefficients and apparent affinities of the glucose facilitated diffusion system of *C. utilis* cells grown in batch culture with 2% (w/v) glucose

The mean and standard deviation of three independent experiments are given. Initial transport rate was measured with $[^3\text{H}]$ glucose, varying the sugar concentration between 0.125 and 500 mM. Hill and Eadie-Hofstee equations were applied to calculate the parameters.

Glucose concn in the transport assay (mM)	Hill coefficient	Apparent affinity (mM)
0.125–7.5	0.9 ± 0.03	2.0 ± 1.2
7.5–40	0.3 ± 0.07	—
40–500	1.0 ± 0.02	76 ± 40

permease would be in the higher-affinity state, whereas at concentrations higher than 40 mM all would be in the low-affinity state. At intermediate concentrations a mixture of states would coexist. Table 2 summarizes the conditions and kinetic parameters of both states.

DISCUSSION

Candida utilis grown in chemostat and batch culture displays a set of transport systems for glucose in what seems to be a general pattern among several yeasts: *Candida wickerhamii* (Spencer-Martins & van Uden, 1985), *Candida sheatae* (Lucas & van Uden, 1986), *Candida intermedia* (Loureiro-Días, 1987), *Pichia ohmeri* (Verma *et al.*, 1987) and *Kluyveromyces marxianus* (Gasnier, 1987). In all these yeasts two systems were found: a H^+ symport when there is no, or a very low concentration of, glucose in the growth medium, and a facilitated diffusion system in the presence of higher concentrations of the sugar.

In *C. utilis*, we found a H^+ symport displaying simple hyperbolic kinetics and a facilitated diffusion with a complex behaviour. Both systems could be observed simultaneously in chemostat-grown cells at steady-state glucose concentrations below 10 mM (Fig. 3), although the relative contribution of each system to the total flow of sugar depended on the sugar concentration.

The H^+ symport has such a high affinity (K_m about $15\ \mu M$) that it is saturated under any conditions where glucose can be detected, so it makes sense that its regulation has to be based on the amount of carrier present in the cells (Fig. 5), controlled by induction and repression. It is also conceivable that the H^+ symport may be inactivated by glucose or even interconverted into a facilitated diffusion system, as previously described in other yeasts (Spencer-Martins & van Uden, 1985; Verma *et al.*, 1987). In contrast, according to our results, regulation of transport at high glucose concentrations might be achieved by changes in the affinity of a single carrier (Fig. 7), although the possibility that two systems might be present should not be discarded.

In *Saccharomyces cerevisiae*, biphasic Eadie-Hofstee plots for initial transport of glucose have also been described (Bisson & Fraenkel, 1984; Busturia & Lagunas, 1986). High-affinity facilitated diffusion has been shown to depend on the presence of some of the glucose-phosphorylating enzymes of this yeast. Current work in one of our laboratories is trying to identify these types of enzyme in *C. utilis*, looking also for kinase-negative mutants. On the other hand, the existence of low-affinity facilitated diffusion in yeasts needs to be re-evaluated, measuring transport at higher concentrations of glucose than those usually used.

If there is a single carrier for facilitated diffusion in *C. utilis*, it remains to be elucidated if its changes in affinity are due to allosteric mechanisms involving a complex permease with several subunits, or to hysteretic behaviour of a monomeric permease. The allosteric mechanism was suggested some years ago (Barnett & Sims, 1976), based on kinetic experiments. The isolation of the permease(s) and/or the corresponding gene(s) seems the only way to solve this problem.

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