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Characterization of 2-deoxyglucose and 6-deoxyglucose transport in *Kluyveromyces marxianus*: evidence for two different transport mechanisms

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Transport of 2-deoxy-D-glucose (2-dGlc) and 6-deoxy-D-glucose (6-dGlc) is studied in *Kluyveromyces marxianus*, grown under different conditions. It is shown that early stationary phase cells contain only one glucose transporter, with low affinity for 6-dGlc and high affinity for 2-dGlc. This transporter is recognized by glucose and fructose. In late stationary phase cells, two transport systems are operative for 6-dGlc, one with a high and one with a low affinity. The high-affinity system appears to be a glucose-galactose carrier, catalyzing uphill transport, energized by coupling sugar transport to translocation of protons. Induction (or derepression) of the high-affinity 6-dGlc transport seems to be coupled, in an as yet unknown way, to citrate consumption and a strong alkalinization of the medium during growth. It is concluded that glucose transport in *K. marxianus* can proceed by at least two mechanisms: a glucose-fructose carrier, probably having phosphotransferase characteristics, and a derepressible glucose/galactose-proton symporter.

Introduction

Transport of 2-deoxy-D-glucose in *Kluyveromyces marxianus* appears to proceed according to a transport-associated phosphorylation mechanism. As proposed for the glucose transport in *Saccharomyces cerevisiae* by Van Steveninck and co-workers [1,2], this mechanism involves phosphorylation of the sugar at the expense of peripherally localized polyphosphate. The evidence in *K. marxianus* was based on the finding that the first molecular species entering the cell is sugar phosphate [3,4]. Moreover, it was shown that, under anaerobic conditions, the amount of sugar phosphate formed (about 2.5–3 μmol/g yeast) originated from cellular polyphosphates [4]. Finally, several lines of evidence suggested the localization of an amount of polyphosphate in the periplasmic space [5–7], with a pool size of about 2.5 μmol/g yeast.

However, apparently conflicting with the proposed mechanism was the finding that 2-dGlc influx seemed to be coupled to influx of protons [8,9], and that sugar uptake caused membrane depolarization [10], even though a proton symport mechanism appeared to be unlikely.

Also in *S. cerevisiae*, the glucose phosphotransferase has been questioned [11–13], even though the pulse kinetics of 2-dGlc uptake showed a similar behavior to that of *K. marxianus*, i.e., sugar phosphate appears first in the cell [14,15]. Recently, evidence has been presented to show that hexokinases could be directly involved in the glucose transport process [16,17].

Most of the experiments mentioned before were

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Abbreviations: 2-dGlc, 2-deoxy-D-glucose; 6-dGlc, 6-deoxy-D-glucose; CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

carried out with glucose analogues that could be phosphorylated, like 2-dGlc. The fact that recently an analogue became available that cannot be phosphorylated (6-deoxy-D-glucose) offered new possibilities for the study of glucose transport [13,18]. The main findings in *S. cerevisiae* were that the sugar cannot be accumulated [13,18,19] and that probably hexokinases influence the transport process [18].

In the present study, transport of 6-dGlc is studied in *K. marxianus*, and is compared with 2-dGlc uptake.

Materials and Methods

K. marxianus CBS 397 was grown in a medium containing, per liter: 1.2 g KH_2PO_4, 2.4 g citric acid, 12 g tripotassium citrate; 3 g $(NH_4)_2SO_4$, 0.6 g $MgSO_4 \cdot 7H_2O$, 1 g KCl, 0.3 g $CaCl_2 \cdot 2H_2O$, 17.5 mg $FeCl_3 \cdot 6H_2O$, 6.25 mg $MnSO_4$, 50 mg NaCl, 50 mg $ZnSO_4 \cdot 7H_2O$, 3.5 mg biotin, 6.25 mg inositol, 6.25 mg calcium panthothenate, 6.25 mg thiamin, 6.25 mg pyridoxine, 6.25 mg nicotinic acid, 6.25 mg riboflavin and 20 g glucose (or lactose). The yeast was cultured in a Microferm fermentor (New Brunswick Scientific Co.) with a culture volume of 5 liter, at 29°C, an agitation of 250 rpm and an airflow of 17 liter/min. Cells were incubated from a 150 ml pre-inoculum, grown for 2 days at 29°C in a culture flask. The yeast was harvested by centrifugation and washed three times with distilled water.

The biomass was determined by measuring the weight of the cells in 250 ml samples of culture medium.

Sugar transport was measured, using tritiated deoxyglucose, at 25°C, aerobically, in a 10% (wet weight/vol.) suspension. Samples (0.1 ml) were diluted in 5 ml ice-cold water, and filtered on cellulose-nitrate filters (0.45 μm pore, Schleicher and Schull). After washing the cells the filter was dried, and the radioactivity was determined by scintillation counting, using Picofluor 30 (Packard) scintillation liquid.

Proton fluxes were measured anaerobically essentially as described before [9]. Anaerobiosis was achieved by incubating the cells under a constant stream of argon gas (containing less than 0.3 ppm oxygen).

Extracellular glucose was determined enzymatically, using the hexokinase/glucose-6-phosphate dehydrogenase assay [20]. Extracellular citrate was measured enzymatically by the method of Dagley, using citrate lyase, lactate dehydrogenase and malate dehydrogenase [21].

2-Deoxy-D-[^{3}H]glucose was obtained from Amersham International, and 6-deoxy-D-[^{3}H]glucose from New England Nuclear.

Results

The characteristics of growth of *K. marxianus* on a glucose-containing medium are shown in Fig. 1. The biomass increases exponentially during the first 10 h, and only slowly beyond this time (stationary phase). The pH of the medium reveals two main events in the metabolism of carbon substrates: an acidification coupled to glucose consumption and exponential growth, and a dramatic pH increase on consumption of part of the medium citrate.

Fig. 2 shows the relation between culture age

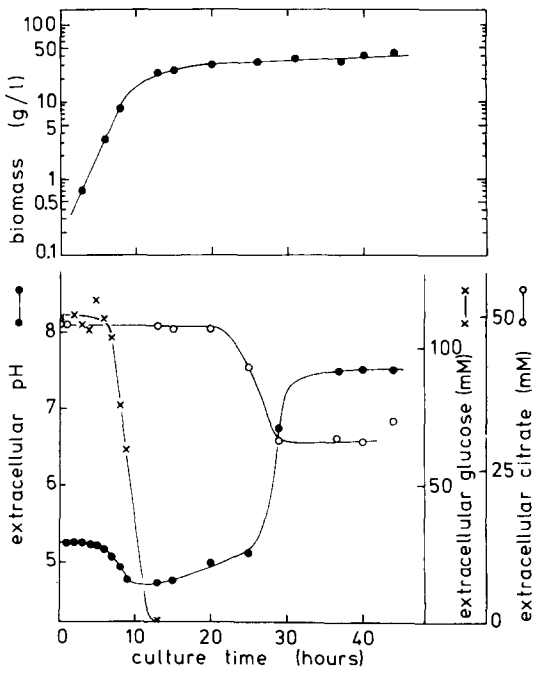

Fig. 1. Characteristics of growth of *K. marxianus* on a glucose-containing medium. The extracellular concentrations and pH are obtained in filtrates of the culture.

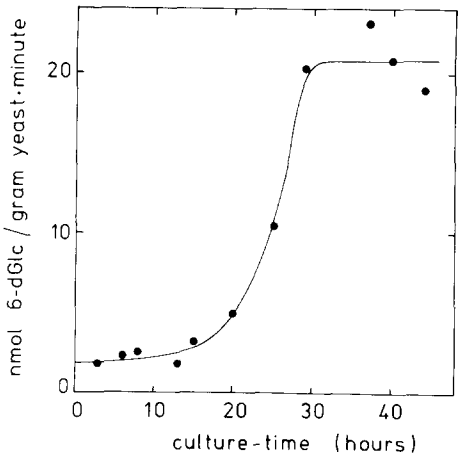

Fig. 2. Influence of culture time on 6-dGlc uptake. Transport was measured in 0.1 M Tris-maleate buffer (pH 4.5). Initial extracellular 6-dGlc concentration: 0.1 mM. Sample time: 5 min after start of transport.

and 6-dGlc uptake. It is shown that during the first 15–20 h of growth uptake is slow, whereas afterwards, at the same time at which pH_{out} increases, 6-dGlc transport is enhanced. The difference between 6-dGlc transport in young and older cultures is determined by both kinetic and energetic differences. In 13-h-grown cultures, transport of 6-dGlc, at low concentrations, is much slower than in 38-h-grown cultures: the initial influx in 13-h-grown cells is about 5% of the value in 38-h-grown cells. Moreover, in 13-h cultures uptake does not exceed the diffusion equilibrium, whereas late stationary phase cells accumulate this sugar (to an extent of 10–20-times the extracellular concentration).

The kinetic difference between the two yeast populations is not found with 2-dGlc, since the initial 2-dGlc influx is only 17% lower in 13-h-grown yeast compared to the 38-h-grown cells. This shows either that there are kinetic differences in transport between sugars that can be phosphorylated and the ones that can not, or that the two glucose analogues do not use the same translocator.

To test the first hypothesis, the kinetics of influx was measured in 13-h- and 38-h-grown cells. It is shown in Table I that 2-dGlc is only translocated by high-affinity transport, whereas 6-dGlc uses a low-affinity translocator in the 13-h-grown cells, and both high- and low-affinity transport in 38-h-grown cells (biphasic kinetics).

TABLE I

K_m VALUES OF DEOXYGLUCOSE TRANSPORT

Transport is measured in 0.1 M Tris-maleate (pH 4.5). Uptake velocities were determined from the linear segments during the first 20 s. K_m values were obtained from Eadie-Hofstee plots of initial uptake. In the case of biphasic kinetics (6-dGlc in 38 h grown cells), the data were computer-analyzed to yield two K_m values.

Yeast	Sugar	K_{low} (mM)	K_{high} (mM)
13 h grown	2-dGlc	0.50	–
13 h grown	6-dGlc	–	100
38 h grown	2-dGlc	1.40	–
38 h grown	6-dGlc	0.8	90

It seems likely that the low-affinity 6-dGlc system in both cell types represents the same translocator, whereas the high-affinity 6-dGlc transport system represents a 'derepressed' active transporter.

Table II demonstrates that in 13-h-grown cells influx of 2-dGlc and 6-dGlc is inhibited by glu-

TABLE II

THE INFLUENCE OF SUGARS ON DEOXYGLUCOSE TRANSPORT

Transport was measured in 0.1 M Tris-maleate (pH 4.5). 2- and 6-dGlc transport was assayed at tracer concentration. Inhibition by sugars was performed with 5 mM of the respective sugar, except for 6-dGlc inhibition of 2-dGlc uptake in 13 h grown cells, where 6-dGlc was present at 100 mM. Uptake velocity was followed from the linear section, during the first 18 s of uptake, and the values are presented as a percentage of the control.

Condition	13 h grown yeast		38 h grown yeast	
	6-dGlc	2-dGlc	6-dGlc	2-dGlc
Control	100 (%)	100 (%)	100 (%)	100 (%)
+ Glucose	39	22	3	5
+ Galactose	89	106	3	16
+ Fructose	40	40	92	68
+ Lactose	106	97	95	60
+ Arabinose	92	101	102	61
+ Xylose	94	105	88	91
+ 6-dGlc	–	53	–	36
+ 2-dGlc	33	–	7	–

cose and fructose. Moreover, 2-dGlc inhibits 6-dGlc influx and vice versa (it should be noted that 6-dGlc as inhibitor is used at a 100 mM concentration, equal to its K_m). Thus in 13-h-grown yeast both deoxyglucose analogues utilize the same translocator. In 38-h-grown cells transport of both deoxglucose analogues is more strongly inhibited by glucose, as compared to 13-h-grown yeast. Inhibition of 6-dGlc transport by fructose, on the other hand, is lower. Most striking is the effect of galactose. Whereas galactose does not inhibit deoxyglucose transport in 13-h-grown yeast, influx of both deoxyglucose analogues is strongly inhibited in 38-h-grown cells. This indicates that in 38-h-grown cells the inducible galactose transport system may be involved in the transport of both 2-dGlc and 6-dGlc. This is supported by experiment with lactose-grown cells. These cells, which have induced galactose uptake [22], accumulate 6-dGlc, and transport is selectively inhibited by 10 mM glucose (85% inhibition) and 10 mM galactose (70% inhibition), whereas 10 mM fructose shows only minor influence (10% influence).

As shown before, using 2-deoxy-D-galactose, the galactose transport system seems to be a proton symport [8]. As 6-dGlc cannot be phosphorylated it seems likely that the accumulation in 38 h-grown cells is also caused by proton symport.

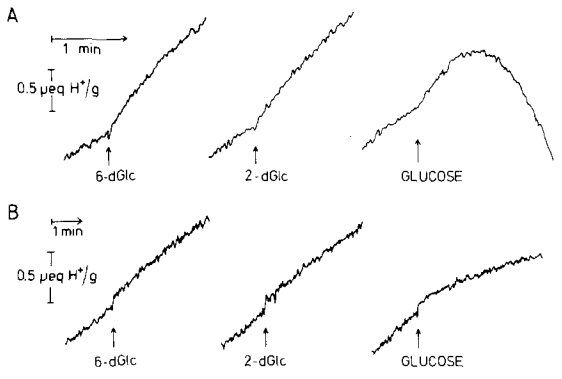

Fig. 3. The influence of sugars on proton fluxes in 13 h grown and 38 h grown yeast. Yeast was suspended in 10% (wet weight/vol.) suspension in 2.5 mM Tris-maleate. Anaerobiosis was achieved by flushing with argon. At the moment of sugar addition pH was 5.8. Additions: 5 mM 6-dGlc, 5 mM 2-dGlc or 2.5 mM glucose. Curves A (upper ones): 38 h grown yeast; curves B (lower ones): 13 h grown yeast. An upward deflection shows pH increase.

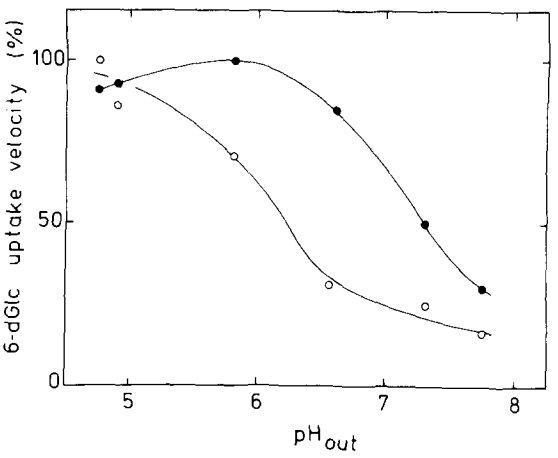

Fig. 4. The influence of external pH on 6-dGlc influx. Transport was measured in 0.1 M Tris-maleate buffer. 6-dGlc is used in tracer concentration. ●: 13 h grown yeast, ○: 38 h grown yeast. Influx velocity is determined from linear uptake during the first 6–18 s.

Support for this view comes from the following experiments. Fig. 3 shows that in 38-h-grown yeast transport of glucose and its analogues stimulates H^+ influx, in contrast to the 13-h-grown cells, which do not reveal sugar-transport-associated H^+ influx. The conditions as shown in Fig. 3, i.e., pH 5.8, were however not stable enough to give reli-

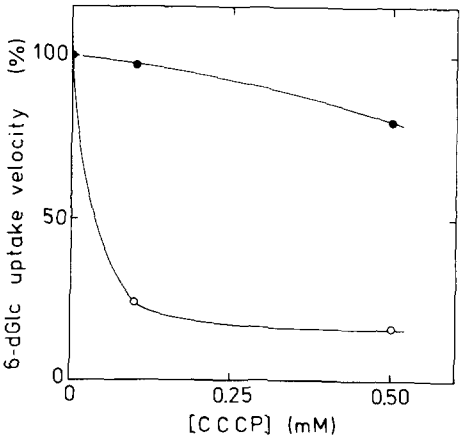

Fig. 5. The influence of CCCP on 6-dGlc influx. Transport was measured in 0.1 M Tris-maleate (pH 4.5). 6-dGlc was used at tracer concentration. ●: 13 h grown yeast; ○: 38 h grown yeast. Influx is determined from the linear uptake during the first 6–18 s. CCCP was preincubated for 2 min before adding 6-dGlc.

able estimates of H^+/sugar stoichiometries. Attempts to measure the reaction at lower pH were impossible in 38-h-grown yeast, because these cells increase the pH of the medium too fast to accurately estimate H^+ influx following sugar transport.

As shown in Fig. 4, 6-dGlc influx in 38-h-grown cells is much more sensitive to the medium pH than 6-dGlc influx in 13-h-grown cells, thus revealing that protons are more closely involved in translocation of 6-dGlc in 38-h-grown cells than in 13-h-grown yeast. Moreover, the pH dependence in 38-h-grown yeast resembles the one found for many H^+ symport systems, having a characteristic pK of approx. 6–6.5 [23–25].

Finally, Fig. 5 indicates that in 38-h-grown yeast the protonophore CCCP, which strongly inhibits H^+ symport in yeast [25], causes a much stronger inhibition of 6-dGlc uptake as compared to 13-h-grown cells. This again makes H^+ symport a likely mechanism for accumulation of 6-dGlc in 38-h-grown cells.

Discussion

It is shown in this paper that 6-dGlc uptake can proceed by both non-equilibrative transport and by active transport, the latter probably being a proton symport. As can be concluded from Table II, it is most likely that 2-dGlc is also transported by these systems: the inhibition by metabolizable sugars is, at low concentration of deoxyglucose, identical for both 2-dGlc and 6-dGlc. Since 13-h-grown cells only show one component (Table I) in this yeast it is clear that translocation proceeds through the same transporter for both glucose derivatives. This carrier recognizes glucose and fructose, and therefore resembles the constitutive glucose-fructose carrier of *S. cerevisiae* [11,26]. In 38-h-grown yeast, 2-dGlc and 6-dGlc influx are mainly glucose- and galactose-sensitive, again showing that, in these cells, both share a common translocator, which catalyzes uphill transport. Under the conditions of Table II, the low-affinity 6-dGlc uptake component (Table I) is not measured in 38-h-grown cells. Also in this population, 2-dGlc appears to be transported via a single high-affinity system (Table I). It seems likely, however, that 2-dGlc transport in 38-h-grown cells also consists of two components, both having high affinity with almost the same K_m values. Firstly, it is probable that low-affinity 6-dGlc uptake in 13-h- and 38-h-grown cells represent the same carrier, probably the constitutive glucose-fructose transporter. The mutual inhibition of both dGlc analogues (Table II) demonstrates that 2-dGlc is transported by this carrier system, which does not recognize galactose. Secondly, Table II shows that 2-dGlc uptake in 38-h-grown cells is less galactose- and more fructose-sensitive than 6-dGlc, showing the existence of a mixed transporter population.

Assuming that inhibition of transport by metabolizable sugars is caused by binding to the transport binding site, followed by membrane translocation of the sugar, it becomes evident that 6-dGlc, and thus also 2-dGlc, can be transported either by a probably constitutive glucose/fructose carrier and by an inducible galactose/glucose active transporter.

The nature of the glucose/fructose carrier can only be speculated upon. This carrier catalyzes transmembrane movement of sugars that can be phosphorylated, like glucose, fructose and 2-dGlc, with high affinity, whereas sugars that cannot be phosphorylated, like 6-dGlc, have low affinity for this carrier. This suggests that for this transport system, phosphorylation seems to be directly involved in the translocation phenomenon, either through the phosphotransferase mechanism, using polyphosphate as phosphoryl source [1,2], or by a hexokinase-coupled uptake system [16–18].

The nature of the glucose/galactose carrier resembles closely a proton symport mechanism. It seems likely from the experiments described here that this particular symporter is identical to the previously described [8,22] inducible galactose carrier. Also for *S. cerevisiae* it has been shown that 6-dGlc is a substrate of the galactose carrier [18,19], even though in that yeast uptake is not accumulative. However, in *S. cerevisiae*, 6-dGlc uptake through the galactose carrier also results in high-affinity transport.

The finding of two components in the glucose transport, one of which is H^+ symport, can explain some previously described results, where it was shown that 2-dGlc uptake caused H^+ influx and membrane depolarization [8–10]. In these

publications the yeast was cultured for 20 h, thus resulting in a cell population containing both glucose transport systems. This explains why H^+ influx in these papers never reached the level of sugar influx (H^+/S ratio < 1), and also why the H^+/S ratio shifted from ± 0.7 at pH 4 to 0 at pH 7 [9] (compare with Fig. 4).

One of the main conclusions of this paper is that, depending on the growth phase of the cells, a transport system can be induced or derepressed. This has been shown before in some yeasts, but mainly based on the control of transport systems by glucose. In these papers it was shown that the presence of high glucose concentration represses, e.g., maltose and galactose uptake in *S. cerevisiae* [27,28]. Moreover, in *Candida wicherhamii* high concentrations of glucose repressed the high-affinity glucose/H^+ symporter and modified it to passive glucose transport [29,30]. In these papers it could be shown that the reverse was also true, i.e., incubation of the cells in the presence of low concentrations of glucose yielded activation of transport systems, or interconversion to H^+ symport. However, the results presented in this paper do not immediately show an effect of glucose. Even a 13-h-grown yeast is cultured almost in the absence of glucose (concentration < 0.5 mM). Moreover, comparison of Figs. 1 and 2 suggests a close relation between induction of high-affinity 6-dGlc uptake, medium pH increase and citrate consumption. It can be speculated that consumption of organic acids induces a cellular metabolism that differs from early stationary phase cells in the sense that glucose/H^+ symport is induced. However, it is as yet unclear what can be the exact trigger for inducing H^+ symport. Further experiments will be necessary to elucidate this mechanism.

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