The role of ATP in the control of H⁺–galactoside symport in the yeast *Kluyveromyces marxianus*

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Transport of methyl β -D-thiogalactoside and *p*-nitrophenyl β -D-galactoside is shown to proceed through the H⁺–lactose symporter of *Kluyveromyces marxianus*. Uptake of these compounds is strongly reduced under anaerobic conditions or aerobically in the presence of antimycin. It is shown that antimycin treatment affects *p*-nitrophenyl β -D-galactoside uptake in a similar way as it affects the cellular amount of ATP, suggesting regulation of *p*-nitrophenyl β -D-galactoside transport by ATP. Also, manipulation of cellular ATP by antimycin treatment followed by glucose incubation, or by aerobic incubation of cells with 2-deoxy-D-glucose, showed a similar dependence of galactoside uptake on the ATP level. Transport of the lipophilic cation tetraphenylphosphonium is affected by ATP variations in a similar way as galactoside influx. It is concluded that ATP regulates H⁺–galactoside symport by its influence on charge translocation. It is discussed that a membrane ATPase probably plays a central role in the control of the activity of H⁺–sugar symport.

INTRODUCTION

Transport of amino acids and sugars by yeasts appears to be extremely sensitive to the oxygen level of the suspension. It was found that active transport of amino acids in *Saccharomyces cerevisiae* was decreased in anaerobic conditions, resulting in a decreased accumulation (Ballarin-Denti *et al.*, 1984; Horák & Kotyk, 1986) or even, in some cases, in a lack of uphill transport (Kotyk *et al.*, 1971).

The same has been observed with carbohydrate transport in some yeasts, where it was also found that accumulation of the substrates was reduced (Kotyk & Höfer, 1965) or even absent (Höfer, 1971; Barnett & Sims, 1982) under anaerobic conditions. Moreover, the uptake velocity of sugars is decreased strongly in oxygen-deprived suspensions (Sims *et al.*, 1984). So, apparently solute transport in a number of yeast strains is tightly coupled to cellular metabolism and therefore relies strongly on the energy state of the cell (see also Sims & Barnett, 1978). Thus far, however, the mechanism of this effect has not been understood very well. The suggestion has been made that mitochondrial ATP plays a role in the phenomenon (Sims *et al.*, 1984). This could also be concluded from the similarity in the effect of anaerobiosis and inhibitors of oxidative phosphorylation on solute transport (Janda, 1979; Barnett & Sims, 1982; Sims *et al.*, 1984). Nevertheless, the proposed role of ATP is not generally applicable, since measurements of proline uptake in *S. cerevisiae* did not show a relation between ATP and substrate accumulation (Horák & Kotyk, 1986).

In the present paper the role of anaerobiosis on galactoside uptake in *Kluyveromyces marxianus* is studied in relation to the possible involvement of ATP in the control of this transport.

EXPERIMENTAL

Kluyveromyces marxianus CBS 397 was grown on 2% lactose for 20 h, and was subsequently harvested and washed as described by Jaspers & Van Steveninck (1976).

Transport was measured at 25 °C in a 2% (wet wt./vol.) yeast suspension buffered with 50 or 100 mM-Tris/maleate at pH 4.5. Uptake of TMG was determined by incubating cells with ¹⁴C-labelled sugar, and filtering aliquots of the suspension, after dilution in ice-cold water, on cellulose nitrate filters (Schleicher and Schüll; 0.45 μ m pore size). After washing with ice-cold water, the filters were dried and the radioactivity measured by liquid-scintillation counting. NPG influx was measured from the release of *p*-nitrophenol after intracellular cleavage of the galactoside (Sims *et al.*, 1984). Samples (0.5 ml) were added to 0.5 ml of 96% (v/v) ethanol. After centrifugation for 1 min in an Eppendorf minicentrifuge, 0.8 ml of supernatant was mixed with 2 ml of 50 mM-NaOH, and the absorbance was measured at 405 nm. Calibration was achieved by measuring standard amounts of *p*-nitrophenol. Transport of TPP was determined as described previously (Van den Broek *et al.*, 1982). The intracellular concentrations were calculated by using a value for the cell water of 0.45 ml/g wet wt. of yeast (Van den Broek *et al.*, 1982).

Aerobic conditions were obtained by bubbling air through the suspension, whereas anaerobiosis was achieved by flushing the suspension in a vessel, closed with a silicon stopper, with highly pure argon gas (purity > 99.9997%; oxygen < 0.3 p.p.m.). Through the narrow gas outlet additions were made from N₂-flushed solutions, and samples were drawn with gas-tight Hamilton syringes.

ATP was measured by the firefly luciferin/luciferase

Abbreviations used: NPG, *p*-nitrophenyl β -D-galactoside; TMG, methyl β -D-thiogalactoside; TPP, tetraphenylphosphonium; $\Delta\psi$, membrane potential; Δ pH, transmembrane pH gradient; K_m , apparent affinity constant; V_{max} , apparent maximal transport velocity.

Fig. 1. Effect of anaerobiosis and antimycin treatment on transport of TMG by *K. marxianus*

Yeast was incubated in 100 mM-Tris/maleate, pH 4.5, under aerobic conditions (●), aerobic conditions in the presence of 2 μ g of antimycin/ml (■) or anaerobic conditions (○). In case of antimycin treatment the yeast was preincubated for 5 min before adding TMG. Initial TMG concentration was 3 μ M. The broken line indicates diffusion equilibrium.

Fig. 2. Effect of anaerobiosis and antimycin treatment on the kinetics of NPG influx

Yeast was incubated in 50 mM-Tris/maleate, pH 4.5, under aerobic conditions (●), aerobic conditions in the presence of 2 μ g of antimycin/ml (■) or anaerobic conditions (○). NPG influx was determined from the linear part of uptake during the first minutes. Antimycin (AA) was preincubated for 5 min. O₂ and Ar represent respectively aerobic and anaerobic incubation. K_m values are given in mM, V_{max} in μ mol of NPG/min per g.

assay as described by Addanki *et al.* (1966) using Sigma FLE-50 firefly lantern extract.

The intracellular pH was determined by the method of Borst-Pauwels & Dobbeltmann (1972).

[¹⁴C]TPP was obtained from Amersham International and [¹⁴C]methyl β -D-thiogalactoside from New England Nuclear. Antimycin A was supplied by Sigma.

RESULTS

The activity of the H⁺-lactose symporter of *K. marxianus* can be determined by using the artificial substrate TMG (Barnett & Sims, 1982; Van den Broek & Van Steveninck, 1982). As shown by Sims *et al.* (1984), it is also possible to utilize nitrophenyl-labelled analogues, such as NPG, for probing lactose transport. These compounds are hydrolysed intracellularly by β -galactosidase to galactose and *p*-nitrophenol. The rate of *p*-nitrophenol formation, measured spectrophotometrically, relates to the activity of the transporter.

Also in the yeast used in the present study it could be shown that NPG is translocated through the H⁺-lactose symporter. Evidence for this conclusion comes from experiments showing that transport is inducible and sensitive to uncouplers and depolarizing agents such as TPP (see also Van den Broek & Van Steveninck, 1982). Moreover, lactose inhibited transport competitively with a K_i of about 2.9 mM, which is close to the K_m values found for lactose fermentation (2 mM) (De la Fuente & Sols, 1962) and for lactose transport (2.8 mM) in a related yeast (Dickson & Barr, 1983). Furthermore, TMG uptake (K_m about 50 mM) was competitively inhibited by NPG with a K_i of 1.5 mM whereas NPG

uptake (K_m about 2.8 mM) was competitively inhibited by TMG (K_i about 60 mM), thus establishing that both substrates use the same transport system.

The results presented in Fig. 1 show that under anaerobic conditions influx of TMG is reduced strongly as compared with transport under aerobic conditions. The same was observed with NPG as a substrate. It is evident however that the cells are still capable of accumulating TMG, a sugar that cannot be phosphorylated, but nevertheless intracellularly exceeds the diffusion equilibrium by at least a factor of 4. The effect of anaerobiosis can, according to Fig. 1, be simulated by incubating the cells with antimycin A, a drug that is an inhibitor of the mitochondrial oxidative phosphorylation (Slater, 1973). This suggests that the effect of anaerobiosis is due to a reduction of the mitochondrial oxidative activity.

Fig. 2 shows the effect of anaerobiosis on the kinetics of NPG uptake. It is found that the apparent affinity constant is hardly sensitive to the oxygen level of the suspension, whereas the maximal transport velocity changes drastically (about 10-fold). The fact that antimycin incubation again closely simulates anaerobic conditions confirms the previous conclusion about the role of mitochondria (see also Sims *et al.*, 1984). Because of this it is likely that ATP is involved in the effect of anaerobiosis. This is confirmed by the experiments presented in Fig. 3. Titration of the cells with antimycin reduced both NPG influx and the cellular ATP concentration (Fig. 3a). A replot of these data, together with data obtained anaerobically and aerobically in the absence of antimycin, indicates a close relation between these two parameters (Fig. 3b). A similar relation between ATP and sugar influx was obtained with TMG.

The ATP depletion induced by anaerobic conditions

Fig. 3. Influence of antimycin on cellular ATP levels and NPG influx

(a) Yeast in 50 mM-Tris/maleate, pH 4.5, was preincubated for 5 min with antimycin. Subsequently transport was analysed from the linear part of uptake during the first minutes. ATP was assayed in samples taken in the same period. Initial NPG concentration was 1 mM. ●, NPG influx; ○, ATP concentration. (b) The relation between ATP level and NPG influx, obtained from the antimycin titrations shown in (a). Values obtained aerobically in the absence of antimycin are indicated (○).

Fig. 4. Influence of glucose preincubation on the ATP levels and NPG influx of antimycin-treated cells

(a) Yeast in 100 mM-Tris/maleate, pH 4.5, was incubated with antimycin (2 μg/ml). After 5 min glucose (50 mM) was added. The indicated glucose preincubation was followed by the assay of NPG uptake (from a 1 mM concentration in the medium) and ATP level as described for Fig. 3. ●, NPG uptake; ○, ATP level. (b) The relation between ATP level and NPG influx, obtained from the glucose preincubations shown in (a).

or antimycin treatment can be partly reversed by stimulation of glycolysis by a suitable substrate. As shown in Fig. 4(a) addition of glucose to an antimycin-treated yeast suspension causes a time-dependent increase of the ATP concentration, with a concomitant increase of NPG uptake. A replot of these data (Fig. 4b) yielded a similar relationship between the ATP level and NPG uptake as found via antimycin titration (Fig. 3b). Kinetic analysis of NPG transport in antimycin-treated cells in the presence and absence of glucose is shown in Fig. 5. When glucose and NPG were added simultaneously, glucose had no influence on the V_{max} of NPG transport, whereas the K_m value was slightly increased

from 1.2 to 2.1 mM, apparently by competition of glucose for the NPG transporter ($K_i > 100$ mM). When glucose was added 45 min before NPG, the apparent K_m value of NPG transport was again 2.0 mM, whereas the V_{max} was increased from 0.6 to 2.3 μmol/min per g. Concomitantly, the cellular ATP concentration was increased from 0.10 (immediately after addition of glucose) to 0.45 μmol/g (after 45 min). Thus, manipulation of cellular ATP either by antimycin incubation, anaerobiosis or stimulation of glycolysis in antimycin-poisoned cells resulted in an alteration of V_{max} , with a constant K_m .

A third method to change the ATP levels was to

Fig. 5. Effect of glucose incubation on NPG uptake kinetics in antimycin-treated cells

Cells were treated as described in Fig. 4. ■, Control without glucose; ○, glucose added simultaneously with starting NPG uptake (0 min Glc); ●, 45 min preincubation with glucose (45 min Glc). K_m values are given in mM, V_{max} in μmol of NPG/min per g.

incubate the yeast aerobically with 10 mM-2-deoxy-D-glucose for times up to 1 h. Subsequent analysis of the amounts of ATP and the NPG uptake revealed a relation similar to that shown in Figs. 3(b) and 4(b), thus establishing the involvement of ATP in the regulation of the H^+ -lactose symport system in *K. marxianus*.

To explain the action of ATP on symport, several mechanisms are conceivable. For instance, ATP might influence symport by affecting the protonmotive force. This was tested by measuring the magnitude of its components in cells with varying ATP concentrations. It was found that inhibition of oxidative phosphorylation

did not result in substantial differences in ΔpH . At constant external pH (=4.9) the inner pH, measured 30 and 75 min after starting the incubation, was 6.6 for aerobic cells and 6.3 for cells incubated anaerobically, or aerobically in the presence of antimycin (pH_{in} at time zero was 6.6). Therefore ΔpH decreases anaerobically only by about 15–20% (from 1.7 to 1.4), which is close to the data found for *S. cerevisiae* (Horák & Kotyk, 1986) and insufficient to explain the observed effects on proton symport. The possible role of the membrane potential was examined by determining $\Delta\psi$ with TPP. This probe of $\Delta\psi$ (see Hauer & Höfer, 1978) is taken up by this yeast at a very low rate. As in some other yeasts (Boxman *et al.*, 1982), equilibration takes many hours. Under anaerobic conditions after 6 h of TPP uptake the steady state was not yet achieved. Therefore, a quantitative analysis of $\Delta\psi$ is impossible. However, Komor & Tanner (1976) showed that it is possible to obtain a qualitative estimate of $\Delta\psi$ by measuring the initial transport velocity of the probe. The experiments of Fig. 6(a) indicate that TPP influx is sensitive to antimycin in a similar way as the cellular ATP concentration. Replot of the data (Fig. 6b) reveals that the TPP uptake velocity and thus $\Delta\psi$ is probably directly related to cellular ATP. This is confirmed by the experiments of Fig. 7, in which the ATP concentration was varied by addition of glucose to antimycin-poisoned cells. Again the TPP uptake velocity and the cellular ATP concentration were clearly inter-related.

DISCUSSION

It has been suggested in a number of publications that active sugar transport in yeast can depend in some way on the amount of ATP in the cell. Support for this suggestion comes from experiments showing that sugar transport is strongly reduced under anaerobic conditions or under aerobic conditions in the presence of antimycin (Sims & Barnett, 1978; Barnett & Sims, 1982; Sims *et al.*, 1984). In the present report the effect of anaerobiosis on sugar transport is confirmed. However, in some respects our results are not in accordance with those of Barnett

Fig. 6. Influence of antimycin on the cellular ATP level and TPP influx

(a) The conditions are those of Fig. 3. Initial TPP concentration was 2 μM . The TPP influx was determined from samples taken during the first 2 min of uptake. ●, TPP influx; ○, ATP level. (b) The relation between ATP level and TPP influx obtained from (a).

Fig. 7. Influence of glucose preincubation on the cellular ATP level and the TPP influx in antimycin-treated cells

(a) The conditions are those of Fig. 4. Initial TPP concentration was $2 \mu M$. TPP influx was followed from samples taken during the first 2 min of uptake. ●, TPP influx; ○, ATP level. (b) The relation between ATP level and TPP influx obtained from results of the experiments shown in (a).

& Sims (1982). These authors reported that under anaerobic conditions galactoside uptake did not exceed the diffusion equilibrium, whereas the present results indicate at least a 4-fold accumulation (Fig. 1). Moreover, Sims *et al.* (1984) reported a K_m change of galactoside transport under anaerobic conditions, whereas the present study reveals, contrarily, a V_{max} change. Considering the high purity of the argon used in this work, these discrepancies cannot be ascribed to incomplete anaerobiosis. Probably these discrepancies are caused by differences in yeast strains and/or growth conditions.

The main conclusion from this report is that ATP regulates the activity of the H^+ -lactose symporter. Moreover, ATP controls the uptake of TPP, showing that the ATP level of the yeast determines the membrane potential. Therefore, it seems highly probable that the effect of ATP on galactoside transport should be explained via this ATP-determined manipulation of the membrane potential, although a concomitant direct allosteric influence of ATP on the symport protein can not be excluded.

It has been indicated before that a plasma membrane H^+ -ATPase is involved in generating a protonmotive force across the cell membrane (Misra & Höfer, 1975; Goffeau & Slayman, 1981). This ATPase catalyses electrogenic translocation of H^+ and maybe K^+ (Villalobo *et al.*, 1981). On the other hand, Sigler *et al.* (1981) showed that acidification of the external medium of *S. cerevisiae* also involves excretion of organic acids and CO_2 , but these processes appear to be electrically silent. The results presented here can best be explained by following the previous suggestions (Eddy & Nowacki, 1971; Misra & Höfer, 1975) that the plasma membrane ATPase is involved in energizing H^+ -sugar symport. It extends these suggestions by showing directly that variation in the amount of the energy-donating substrate of the ATPase induces a direct effect on symport.

Further, the experimental evidence presented demonstrates that this effect is caused by creating a membrane potential which is directly linked to the amount of ATP. This latter result is analogous to the one of Slayman *et al.* (1973), obtained with the ATPase of *Neurospora crassa*. They showed that $\Delta\psi$ in this fungus relates to the ATP concentration.

Recently, Serrano *et al.* (1986) showed that a functional plasma membrane ATPase is required for cell viability of yeast. They suggested that this enzyme is necessary for active nutrient uptake. The findings of this paper support the view that the activity of this enzyme is essential for electrogenic transport.

Finally, the observed influence of $\Delta\psi$ on H^+ symport is expressed in a change of V_{max} , and not of K_m . In a previous report (Van den Broek & Van Steveninck, 1982) it was shown that TMG uptake probably proceeds via a so-called non-mobile carrier (which is a carrier with apparently fixed substrate-binding sites at each side of the membrane). On theoretical grounds it could be predicted that the V_{max} , but not the K_m , of such a non-mobile carrier will be affected by the membrane potential (Van den Broek & Van Steveninck, 1982). Thus the observed $\Delta\psi$ -induced change of V_{max} , without a concomitant change of K_m (see Figs. 2 and 5) is in accordance with the previously presented model of galactoside transport in this yeast strain.

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