Biochimica et Biophysica Acta, 693 (1982) 213–220
Elsevier Biomedical Press

BBA 71462

KINETIC ANALYSIS OF H^+/METHYL β-D-THIOGALACTOSIDE SYMPORT IN *SACCHAROMYCES FRAGILIS*

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(Received June 15th, 1982)

Key words: Kinetic analysis; Proton-methyl β-D-thiogalactoside symport; Hexose transport; Membrane potential; (S. fragilis)

A theoretical description of initial uptake kinetics of H^+/sugar symport is given, with emphasis on the differences between carrier and non-carrier systems. Transport of methyl β-D-thiogalactoside in *Saccharomyces fragilis* is shown to proceed via the inducible lactose transporter. Uptake of this sugar stimulates electrogenic H^+ influx. Together with the correlation between methyl β-D-thiogalactoside accumulation and the proton-motive force this shows that transport proceeds via H^+ symport. Kinetic analysis of initial influx revealed that transport proceeds via a single transport system, sensitive to changes in membrane potential. The pH dependence of the kinetic parameters showed that K_{app} is almost pH insensitive, whereas V_{app} decreases strongly at increasing extracellular pH. It is shown that transport proceeds, most likely, via a non-carrier system, with random binding of H^+ and sugar, in a system where binding of the first ligand does not influence binding of the second.

Introduction

Uphill transport of solutes often proceeds via cotransport with, for example, protons. In this way accumulation of these compounds is brought about by the direct thermodynamic coupling between the electrochemical gradients of both substrates [1–3]. On the molecular level, several studies have been performed in order to elucidate the mechanism of action of the transport system. Analysis of initial uptake kinetics can give an indication of the sequence of binding of the two substrates.

Using this method, for a number of solutes obligatory ordered binding has been shown, with H^+ binding first to the translocator. For example, sulfate uptake in *Penicillium* [4] and yeast [5] as well as phosphate transport in *Neurospora* [6] requires primarily the binding of H^+, which supposedly results in an increase of the affinity of the translocator for the solute to be transported.

It has been suggested that the same ordered sequence applies to several H^+/sugar symporters in *Escherichia coli* [7,8].

On the other hand H^+/sulfate symport via the erythrocyte band 3 protein [9,10] exhibits random binding of the ligands to the transporter. In previous studies we demonstrated a similar random binding of H^+ and substrate for sorbose/H^+ symport in *Saccharomyces fragilis* [11]. In this case kinetic analysis was complicated by the presence of a second, passive transport system for sorbose. Therefore the present study was performed on methyl β-D-thiogalactoside for which only one transport mechanism, which appeared to be a H^+-sugar symport system, is operative.

It is shown in this paper that methyl β-D-

Abbreviations: TPP, tetraphenylphosphonium; diS-C_3-(5), 3,3'-dipropylthiadicarbocyanine; $\Delta\psi$, membrane potential; K_{app}, apparent affinity constant; V_{app}, apparent maximal transport velocity.

thiogalactoside is taken up via the inducible H^+/lactose symporter of *S. fragilis*. A study of the initial uptake kinetics showed that transport proceeds after random binding of the ligands.

Further, analysis of the transport kinetics indicates a non-carrier, rather than a mobile carrier system.

Theoretical section

The general model for H^+-substrate symport is shown in Fig. 1. With the assumptions: (1) $S_i = 0$ (initial influx kinetics), (2) the translocator is in equilibrium with ligands in solution, (3) H^+/S stoichiometry is 1, (4) the total amount of ligand binding sites is constant, this model represents a mobile carrier system. This system can be visualized as a carrier molecule with a single set of binding sites (for substrate and proton). These binding sites will be exposed alternately to the outside and the inside of the membrane either by a diffusion-like process ('ferry-boat' carrier) or by a conformational change in a carrier molecule that spans the membrane.

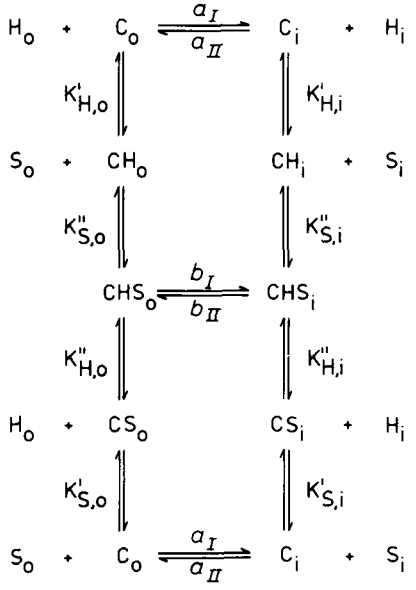

Fig. 1. General transport model, describing H^+-sugar symport. C_o, ..., CHS_o, C_i, ..., CHS_i, ligand binding sites facing, respectively, the outside or inside of the cell; S, sugar; H, proton; $K'_{H,o}$, ·····, $K'_{S,i}$, dissociation constants; a_I, ..., b_{II}, translocation velocity constants.

Defining the dissociation constants:

$$K'_{H,o} = \frac{[C_o][H_o]}{[CH_o]} \quad K''_{H,o} = \frac{[CS_o][H_o]}{[CHS_o]} \quad K'_{S,o} = \frac{[C_o][S_o]}{[CS_o]}$$

$$K''_{S,o} = \frac{[CH_o][S_o]}{[CHS_o]} \quad K'_{H,i} = \frac{[C_i][H_i]}{[CH_i]} \quad \text{etc.,}$$

the total amount of binding sites:

$$C_T = C_o + CH_o + CS_o + CHS_o + C_i + CH_i + CS_i + CHS_i$$

and taking into account the fluxes:

$$J_C = a_I[C_o] - a_{II}[C_i]$$

$$J_{CHS} = \text{net sugar flux} = b_I[CHS_o] - b_{II}[CHS_i]$$

$$\text{with } J_C + J_{CHS} = 0,$$

the kinetic parameters can be derived for the cases of random H^+ and substrate binding to the translocator, random binding with independent binding sites (i.e. $K'_{H,o} = K''_{H,o}$; $K'_{S,o} = K''_{S,o}$ etc.) and of ordered binding, with either H^+ or S binding first. The results are summarized in Table I.

If an additional assumption is made, viz. that the total number of ligand-binding sites at each membrane-solution interface is constant, the model shown in Fig. 1 represents a non-carrier system, as defined a.o. by Borst-Pauwels [12]. On the molecular level such a non-carrier system can be visualized in two ways. In the first place the translocator may span the membrane, with fixed substrate binding sites at each side of the membrane. Transport of the bound substrate follows e.g. by shifting of the substrate molecule to another site, from which it is released at the other side of the membrane (see Ref. 12). On the other hand a mobile carrier system will exhibit the characteristics of a non-carrier system when $a_{II} \gg a_I$ and $a_{II} \gg b_I$ thus, when the return of the empty carrier to the external membrane face is very fast as compared to the velocity of inward movement of the free and substrate-loaded carrier. Under these circumstances the total number of ligand binding sites at each membrane interface will remain constant under all conditions of initial influx.

The kinetic constants for this non-carrier system, as derived in a previous paper [11] are summarized in Table II. As shown before [11] this

TABLE I

THE KINETIC PARAMETERS FOR H^+ SYMPORT IN A CARRIER MODEL DURING INITIAL INFLUX ($S_i = 0$)

	K_{app}	V_{app}
Random	$\frac{K'_{S,o}K''_{H,o}(a_{II}K'_{H,i}(K'_{H,o}+H_o)+a_IK'_{H,o}(K'_{H,i}+H_i))}{K'_{H,o}(a_{II}K'_{H,i}(K''_{H,o}+H_o)+b_IH_o(K'_{H,i}+H_i))}$	$\frac{a_{II}b_IC_TK'_{H,i}H_o}{(a_{II}K'_{H,i}(K''_{H,o}+H_o)+b_IH_o(K'_{H,i}+H_i))}$
Random, with independent binding sites	$\frac{K'_{S,o}(a_{II}K'_{H,i}(K'_{H,o}+H_o)+a_IK'_{H,o}(K'_{H,i}+H_i))}{(a_{II}K'_{H,i}(K'_{H,o}+H_o)+b_IH_o(K'_{H,i}+H_i))}$	$\frac{a_{II}b_IC_TK'_{H,i}H_o}{(a_{II}K'_{H,i}(K'_{H,o}+H_o)+b_IH_o(K'_{H,i}+H_i))}$
H^+ binds first	$\frac{K''_{S,o}(a_{II}K'_{H,i}(K'_{H,o}+H_o)+a_IK'_{H,o}(K'_{H,i}+H_i))}{H_o(a_{II}K'_{H,i}+b_I(K'_{H,i}+H_i))}$	$\frac{a_{II}b_IC_TK'_{H,i}}{(a_{II}K'_{H,i}+b_I(K'_{H,i}+H_i))}$
S binds first	$\frac{K'_{S,o}K''_{H,o}(a_I+a_{II})}{(a_{II}(K''_{H,o}+H_o)+b_IH_o)}$	$\frac{a_{II}b_IC_TH_o}{(a_{II}(K''_{H,o}+H_o)+b_IH_o))}$

non-carrier model applies to H^+/sorbose symport in *S. fragilis*. It should be noted that the constants, summarized in Table I indeed reduce to those in Table II when $a_{II} \gg a_I$ and $a_{II} \gg b_I$.

As discussed in several publications [13–15], it may be expected that the membrane potential can exert an influence on the magnitude of flux constants a_I, a_{II}, b_I and b_{II}. Association of the ligands to the carrier is supposed to be much less sensitive to $\Delta\psi$. Assuming a symmetric Eyring free energy barrier combined with a linear fall of the membrane potential through the membrane [16] this results in $\Delta\psi$-dependent rate constants.

$a_I = a_I^* \cdot y^{z_C}$ $\quad a_{II} = a_{II}^* \cdot y^{-z_C}$

$b_I = b_I^* \cdot y^{z_C+z_H}$ $\quad b_{II} = b_{II}^* \cdot y^{-(z_C+z_H)}$

TABLE II

THE KINETIC PARAMETERS FOR H^+ SYMPORT IN A NON-CARRIER MODEL DURING INITIAL INFLUX ($S_i = 0$)

	K_{app}	V_{app}
Random	$\frac{K''_{H,o}K'_{S,o}(K'_{H,o}+H_o)}{K'_{H,o}(K''_{H,o}+H_o)}$	$\frac{b_IC_TH_o}{K''_{H,o}+H_o}$
Random, with independent binding sites	$K'_{S,o}$	$\frac{b_IC_TH_o}{K'_{H,o}+H_o}$
H^+ binds first	$\frac{K''_{S,o}(K'_{H,o}+H_o)}{H_o}$	b_IC_T
S binds first	$\frac{K''_{H,o}K'_{S,o}}{K''_{H,o}+H_o}$	$\frac{b_IC_TH_o}{K''_{H,o}+H_o}$

with $y = \exp(-F \cdot \Delta\psi \cdot \eta / 2\,RT)$,

η = relative distance of transmembrane movement of the electrically charged group,

$a_I^*, a_{II}^* \ldots = a_I, a_{II} \ldots$ at $\Delta\psi = 0$,
z_C = charge of carrier,
z_H = charge of proton,
R, T, F have their usual meaning.

Substitution of these terms in the equation of Tables I and II, at constant pH_{in} and pH_{out} yields corrected equations as shown in Table III, where A, B, C, D and E are composite constants, different for the four possible reacting types of the transporter (random or ordered binding). Apparently this yields criteria, which can be utilized to discriminate between carrier and non-carrier

TABLE III

THE DEPENDENCE OF THE KINETIC PARAMETERS FOR H^+ SYMPORT ON THE MEMBRANE POTENTIAL, AT CONSTANT pH_{out} AND pH_{in}

The charge of S is taken to be zero. A, B, ..., E are complex constants. The meaning of y is explained in the text.

	K_{app}	V_{app}
Carrier	$\frac{A+B \cdot y^{2z_C}}{1+C \cdot y^{(2z_C+z_H)}}$	$\frac{D \cdot y^{(z_C+z_H)}}{1+C \cdot y^{(2z_C+z_H)}}$
Non-carrier	Constant	$E \cdot y^{(z_H+z_C)}$

systems. For instance, in a non-carrier system K_{app} appears to be independent of $\Delta\psi$ whereas V_{app} is only sensitive to $\Delta\psi$ when $z_C + z_H$ deviates from zero. For the carrier system, on the other hand, K_{app} and V_{app} will, irrespective the value of z_C, always be more or less sensitive to changes of the $\Delta\psi$. Further, solving the constants A, B, C and D in Table III on the basis of the data presented in Table I, exhibits a pH_{in}-dependence of the kinetic constants of the carrier system when studying initial influx, except for the situation where S binds first, whereas for the non-carrier system the kinetic constants are independent of pH_{in}. Finally in case of random binding, with independent sites, the K_{app} of the non-carrier will be insensitive to changes of pH_{out}, whereas the carrier will show a dependence on the external pH.

Materials and Methods

Sacharomyces fragilis was grown, with glucose or lactose as carbon source harvested and washed as described before [17].

Sugar transport, following the procedure of Ref. 3, was measured in 10% w/v cell suspensions, in some experiments buffered with 0.2 M Tris-maleate. H^+ fluxes and transmembrane potentials were measured as described before [18,3].

The presence of sugarphosphates in cellular extracts was determined using the conventional Ba-Zn method [19], or by performing descending paper chromatography of cellular extracts, as described earlier [20]. Measurements of the intracellular water space were performed as described before [21] and revealed a value of 0.45 ml/gram yeast (wet weight).

[^{14}C]TPP and [^{14}C]inulin were supplied by the Radiochemical Centre, Amersham and [^{14}C]methyl β-D-thiogalactoside by New England Nuclear. DiS-C_3-(5) iodide was a gift of dr. A.S. Waggoner, Amherst College, Amherst.

Results

Transport of methyl β-D-thiogalactoside by *S. fragilis* appears to be concentrative. As shown in Fig. 2, this yeast can accumulate this sugar, with an initial medium concentration of 1 mM, up to 20 mM intracellularly. The transport velocity is strongly dependent on the growth conditions. As shown in Fig. 2 the influx velocity is highest in cells cultured with lactose as carbon source, suggesting the involvement of a transport system, which is induced by lactose. With glucose- and ethanol-cultured yeast the methyl β-D-thiogalactoside uptake velocities were, respectively, 25% and 10% of the uptake rate in lactose-cultured yeast. Several lines of evidence indicate that methyl β-D-thiogalactoside is transported via the lactose translocator. As shown in Fig. 2, lactose induces

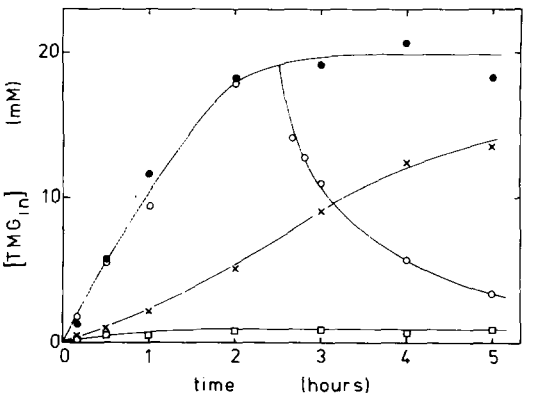

Fig. 2. Uptake of methyl β-D-thiogalactoside (TMG) by *S. fragilis*. 10% yeast is incubated aerobically in 0.2 M Tris-maleate (pH 4.5). ●——●, lactose-cultured yeast; □——□, lactose-cultured yeast in the presence of 1 mM CCCP; ○——○, lactose-cultured yeast with addition of 250 mM lactose after 150 min incubation; ×——×, glucose-grown yeast. Initial TMG concentration: 1 mM.

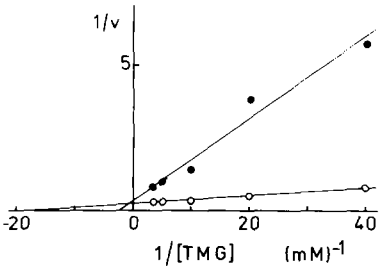

Fig. 3. The influence of lactose on the methyl β-D-thiogalactoside (TMG) uptake velocity. 10% lactose-cultured yeast is incubated aerobically at pH 5. The initial uptake velocity is determined from measurements between 1 and 4 min after methyl β-D-thiogalactoside addition to the yeast. ○——○, control; ●——●, 25 mM lactose is added 0.5 min before methyl β-D-thiogalactoside. $1/v$ is expressed in μmol methyl β-D-thiogalactoside per gram yeast per min.

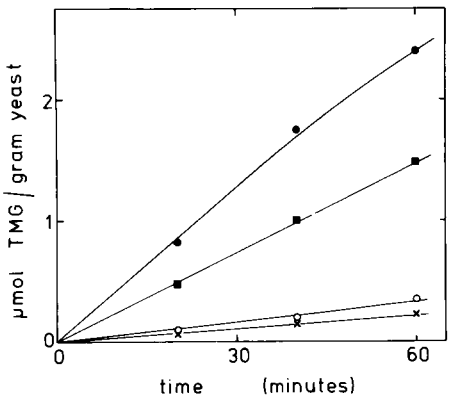

Fig. 4. Induction of the methyl β-D-thiogalactoside(TMG)transport system. Ethanol cultured yeast was incubated aerobically with 2.5% lactose for 1 h (■———■) or 4 h (●———●), with 2.5% glucose for 4 h (×———×) or without extra addition (○———○). After washing the cells three times with distilled water, uptake was measured aerobically in 0.2 M Tris-maleate (pH 4.5). Initial methyl β-D-thiogalactoside concentration 1 mM.

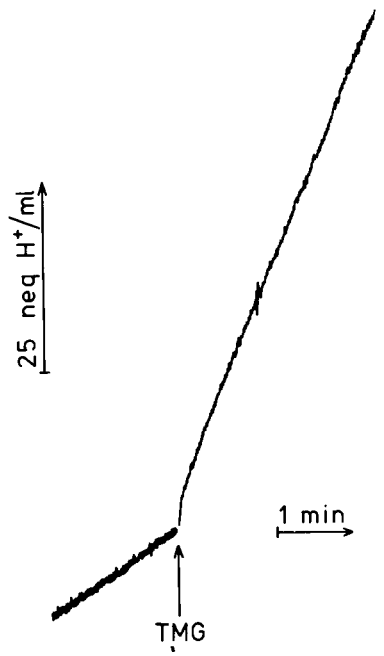

Fig. 5. Stimulation of H^+ influx during methyl β-D-thiogalactoside(TMG) uptake. 10% (w/v) yeast is incubated in 1 mM Tris-maleate. Initial pH 5.4. After recording a baseline, 5 mM methyl β-D-thiogalactoside is added. The curve was calibrated with known amounts of HCl. An upward deflection indicates H^+ influx.

counterflow of accumulated methyl β-D-thiogalactoside. Further, when present in a 250-times molar excess, lactose caused 88%, galactose 49%, glucose 22% and fructose 0% inhibition of methyl β-D-thiogalactoside transport. Finally Fig. 3 shows that inhibition of methyl β-D-thiogalactoside uptake by lactose is competitive. In the presence of 25 mM lactose the K_{app} changes from 64 to 460 mM, whereas V_{app} remains virtually constant.

Induction of the lactose carrier can occur in a few hours. Fig. 4 demonstrates that incubation of ethanol cultured yeast with lactose stimulates methyl β-D-thiogalactoside uptake. This effect is not caused by the input of energy, since glucose does not stimulate the methyl β-D-thiogalactoside uptake velocity.

The high accumulation, coupled to the strong uncoupler sensitivity (Fig. 2), points to the involvement of energy coupled transport. Analysis of cellular extracts with the Ba-Zn reagent or with paper chromatography showed that no phosphorylated products were formed. Therefore the possibility of energy coupling via a phosphotransferase can be rejected. As shown in Fig. 5, influx of methyl β-D-thiogalactoside stimulates H^+ uptake. Under anaerobic conditions the H^+/methyl β-D-thiogalactoside stoichiometry appeared to be 0.87 ± 0.21 at 2.5 mM methyl β-D-thiogalactoside or 0.85 ± 0.26 at 5 mM methyl β-D-thiogalactoside.

Fig. 6 demonstrates that methyl β-D-thiogalactoside as well as lactose-uptake initially de-

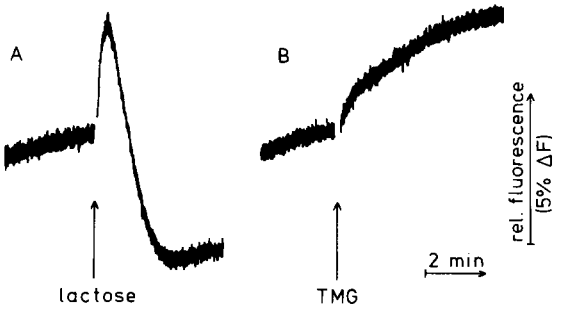

Fig. 6. The influence of lactose and methyl β-D-thiogalactoside (TMG) on diS-C_3-(5) fluorescence in the presence of yeast. Lactose-grown yeast (0.1% w/v) was incubated in 10 mM Tris-maleate (pH 4.5). Addition: (A) 2 mM lactose, (B) 25 mM methyl β-D-thiogalactoside. Fluorescence is expressed in percentage relative to the fluorescence in the absence of yeast. An upward deflection indicates depolarization.

TABLE IV

THE RELATIONSHIP BETWEEN THE PROTON-MOTIVE FORCE ($\Delta\tilde{\mu}_{H^+}$) AND METHYL β-D-THIOGALACTOSIDE ACCUMULATION

The proton-motive force and methyl β-D-thiogalactoside accumulation were measured using the method described earlier [3]. Initial methyl β-D-thiogalactoside concentrations: 0.1 mM.

pH_{out}	$\Delta\tilde{\mu}_{H^+}$ (mV)	Methyl β-D-thiogalactoside accumulation (mV)
4.5	130	114
4.8	124	90
5.9	92	84

polarizes the membrane, indicating electrogenic transport. Finally Table IV shows that the magnitude of the proton-motive force is of the same order as methyl β-D-thiogalactoside accumulation. These observations establish that methyl β-D-thiogalactoside uptake occurs via a sugar-proton symport system.

Kinetic analysis of initial influx resulted in linear Eadie-Hofstee and Lineweaver-Burk plots in a range of 0.1 to 300 mM methyl β-D-thiogalactoside, both at pH 4.5 and 7.5. Therefore it is clear that transport proceeds via only one transport system (see Ref. 11).

Study of the pH dependence of the kinetic parameters demonstrates that V_{app} decreases strongly on increasing the extracellular pH. The apparent affinity (K_{app}), on the other hand, is almost pH insensitive (Fig. 7). Referring to the data in Tables I and II, this indicates that methyl β-D-thiogalactoside uptake proceeds via a non-carrier system with random binding of H^+ and sugar, and more or less independent binding sites.

However, comparing the data from Fig. 7 with simulations as presented in an earlier publication [11], showed that the decrease in V_{app} at high external pH is smaller than expected on the basis of the theoretical model. This indicates that a second factor might be involved. Because of the strong increase in transmembrane potential on rising the pH of the medium [3], it was investigated whether $\Delta\psi$ might influence the transport kinetics. Depolarisation of the plasmamembrane by high concentrations TPP [3] resulted in a strong reduction of the methyl β-D-thiogalactoside influx velocity (Fig. 8), indicating that $\Delta\psi$ indeed exerts an influence on the uptake kinetics of this sugar.

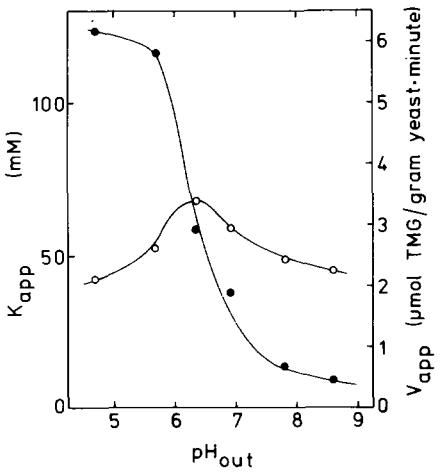

Fig. 7. The influence of the external pH on the kinetic parameters of initial methyl β-D-thiogalactoside (TMG) influx. The initial influx velocity was determined from aerobic uptake experiments between 1 and 4 min after mixing lactose cultured yeast and methyl β-D-thiogalactoside. The kinetic parameters are determined from Eadie-Hofstee plots. ●——●, V_{app}; ○——○, K_{app}.

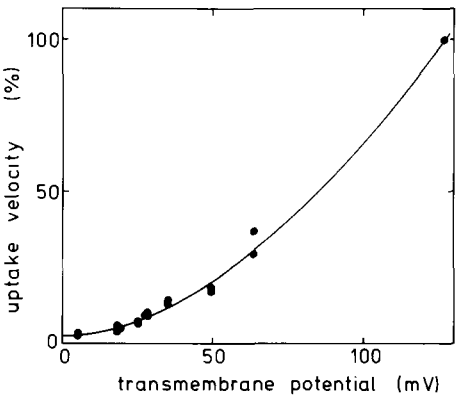

Fig. 8. The influence of the transmembrane potential on the methyl β-D-thiogalactoside uptake velocity. 10% lactose-grown yeast was incubated aerobically in 0.2 M Tris-maleate (pH 5.5). The uptake velocity was determined from measurements between 1 and 4 min after mixing yeast and methyl β-D-thiogalactoside (3.6 μM). The transmembrane potential (negative inside), measured using the TPP distribution, was varied by varying the TPP concentration (between 3.6 μM and 5 mM) in the medium. TPP was added 10 min before methyl β-D-thiogalactoside. The uptake velocity is expressed in percentage relative to the highest value, measured at the lowest TPP concentration.

Discussion

Transport of methyl β-D-thiogalactoside by *Saccharomyces fragilis* occurs via the inducible H^+/lactose symport system, as shown by the experimental results summarized in Figs. 2–6 and Table IV. A study of the initial uptake kinetics revealed a pH independent K_{app}, whereas V_{app} was found to be strongly dependent on the external pH. Referring to Tables I and II, this indicates that transport occurs via a non-carrier system, with random binding of H^+ and sugar to the translocator. Further, binding of one ligand to the translocator appeared to have no influence on the binding of the second ligand (independent sites).

Further it appeared that variation of $\Delta\psi$ (by increasing the medium pH or by using high concentrations TPP) affected methyl β-D-thiogalactoside transport kinetics. Referring to Table III this means that $E \cdot y^{(z_H + z_C)}$ varies with $\Delta\psi$, indicating that $z_H + z_C$ deviates from zero. Probably this implies that z_C, i.e. the charge of the unloaded carrier, will be zero. Association of the proton to the translocator renders it positively charged. This would be in accordance with the earlier suggestions that a histidine-imidazole group might be responsible for proton binding in H^+ symport systems [11,22].

A non-carrier mechanism has also been described by Theuvenet et al. [23] for the Rb^+ transport *Saccharomyces cerevisiae*. This may indicate that, at least in yeast, non-mobile carrier systems are quite common. In this context it should be stressed, however, that a non-carrier system can also be interpreted as a mobile carrier system under the limiting conditions that $a_{II} \gg a_I$ and $a_{II} \gg b_I$ (Fig. 1), as discussed in the theoretical section.

The fact that binding of the ligands seems to proceed randomly, with the restriction that binding of the first ligand does not influence binding of the second, contrasts the mechanism found for many other symport systems where H^+ appears to bind first [4–8]. In these systems binding of H^+ is supposed to induce a conformational change in the transport protein, resulting in an increased affinity for the second ligand. Association of the second ligand subsequently causes a second conformational change, triggering transmembrane movement of the bound substrates. Along similar lines of reasoning the mechanism of methyl β-D-thiogalactoside transport in S. fragilis might comprise a conformational change of only a part of the transport protein on binding of the first ligand (either H^+ or sugar), leaving the binding site for the second ligand unchanged. Regarding the fact that neither the CS nor the CH complex can be translocated over the membrane, association of the second ligand (yielding the CSH complex) is required. This would result in a similar conformational change of a different part of the transporter, thus triggering translocation. Elucidation of the exact mechanism of transport via this type of carrier on the molecular level will require further investigations.

Acknowledgement

Miss Karmi Christianse is greatly acknowledged for carrying out part of the experiments. This study was carried out under auspices of the Netherlands Foundation for Biophysics with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

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