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Na^+-DEPENDENT METHYL β-THIOGALACTOSIDE TRANSPORT IN *SALMONELLA TYPHIMURIUM*

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Summary

We have studied the role of sodium ions in methyl β-thiogalactoside (TMG) transport via the melibiose permease (TMG II) in *Salmonella typhimurium*.

TMG uptake via TMG II in anaerobic, starved and metabolically poisoned cells is dependent on an inward-directed Na^+ gradient.

Cells which have been partially depleted of endogenous substrates show H^+ extrusion upon sodium-stimulated TMG influx.

Measurements of the electrochemical H^+ gradient in cells, starved in different ways for endogenous substrates, suggest that this proton extrusion is probably not linked to the actual translocation mechanism but is the result of metabolism induced by TMG plus Na^+ uptake.

Introduction

The uptake of a number of sugars and amino acids by many animal cells is thought to occur in symport with sodium ions [1]. In contrast, in bacterial cells many solutes are transported by H^+-solute symport systems, which are dependent on the electrochemical H^+ gradient [2]. A few systems have been described in bacteria, however, which require Na^+ for transport activity. For instance, Stock and Roseman [3] demonstrated that transport of methyl β-D-thiogalactoside (TMG) via the melibiose permease (TMG II) in *Salmonella typhimurium* is stimulated by Na^+ or Li^+ and proposed a Na^+-TMG symport. These studies have been extended recently by Tsuchiya et al. [4] and Tokuda and Kaback [5] who concluded that TMG transport via TMG II is driven by an electrochemical Na^+ gradient in both *Escherichia coli* and *S. typhimurium*. Glu-

Abbreviations: αMG, methyl α-D-glucopyranoside; TMG, methyl β-D-thiogalactopyranoside; TMG II, melibiose permease; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

tamate and leucine transport in *Halobacterium halobium* [6,7] and glutamate transport in *E. coli* [8,9] have also been shown to be dependent on a Na^+ gradient. Furthermore, evidence has been presented that these bacteria possess a Na^+-H^+ antiport system [5,10,11] which enables them to maintain a low intracellular sodium concentration under conditions where energy maintains a low intracellular H^+ concentration. Recently we presented some data on the stimulation of TMG transport via TMG II by Na^+ in anaerobic, starved and metabolically poisoned cells of *S. typhimurium* [12]. Surprisingly, we found that the inward movement of TMG via TMG II is accompanied by extrusion of protons. A similar finding has been reported recently by Tsuchiya et al. [4]. Both Na^+-stimulated TMG uptake and TMG-induced H^+ extrusion are sensitive to the uncoupler FCCP, while monensin, which catalyzes an electroneutral exchange of Na^+ for H^+, inhibits H^+ extrusion, but has no effect on TMG uptake [12]. Proton extrusion and the effect of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and monensin are difficult to reconcile with a Na^+-TMG symport system.

In this paper we show that the proton extrusion, observed under conditions when TMG is moving into starved cells of *S. typhimurium*, is probably not directly connected with Na^+-TMG symport. Measurements of the electrochemical H^+ gradient in cells starved in different ways for endogenous substrates suggest that the extrusion of protons is linked to metabolism.

Materials and Methods

Preparation of cells. S. typhimurium strain SB 3507 (*trpB223*) was grown at 37°C to mid-exponential phase in medium A [13], supplemented with 20 μg L-tryptophan per ml and 0.2% melibiose. Cells were depleted of endogenous energy by resuspension in medium A (at half the original volume) and incubation for 1 h at 37°C, either in the absence or in the presence of 20 mM α-methylglucoside, plus 40 mM azide [14]. For the determination of $\Delta\psi$, the starved cells were treated with 120 mM Tris-HCl, containing 0.5 mM EDTA, final pH 8.0, to make them more sensitive to valinomycin [15].

Transport assay. For transport studies, cells were washed twice with a medium containing 25 mM Tris-MOPS, 2 mM KCN, 2 mM Tris-iodoacetate, pH 7.5, and resuspended in the same medium to a concentration of 15—25 mg dry weight per ml. Transport studies were performed as described elsewhere [16]. To keep cells anaerobic, N_2 was bubbled through the cell suspension during the experiment.

pH Measurements. For pH measurements cells were washed twice with a medium containing 200 mM sucrose, 30 mM choline-chloride, 2 mM KCN, 2 mM Tris-MOPS, final pH 7.7, and resuspended in the same medium to a concentration of 15—25 mg dry weight per ml. pH measurements were done as described in ref. 13.

Determination of $\Delta\tilde{\mu}_{H^+}$. $\Delta\tilde{\mu}_{H^+}$, the electrochemical proton gradient, ($\Delta\tilde{\mu}_{H^+}$ = ($\Delta\psi - 59\ \Delta$pH)mV), was determined in EDTA-treated cells in the presence of valinomycin under conditions similar to those of uptake experiments or pH measurements. The ΔpH component was calculated from the distribution of [^{14}C]methylamine (0.06 mM, 0.14 μCi/ml) between the intracellular water

space and the medium [17], whereas the $\Delta\psi$ component was calculated from the distribution of K^+ [18]. ^3H_2O (1.5 μCi/ml) and [^{14}C]inulin (12 μM, 0.14 μCi/ml) served as markers for pellet water and extracellular space, respectively [19]. Samples were centrifuged through silicone oil (Wacker Chemie AR 100) into 15% perchloric acid. Samples from supernatant and perchloric acid extract were counted for both ^{14}C and ^{3}H content. Intra- and extracellular K^+ was measured with a Perkin-Elmer atomic absorption spectrophotometer (Model 305).

ATP determination. ATP was measured in neutralized perchloric acid extracts according to the method described by Williamson and Corkey [20].

Special Chemicals. ^{14}C-Labeled methyl β-D-thiogalactoside (50 μCi/0.21 mg) and [^{14}C]inulin (50 μCi/22.4 mg) were obtained from New England Nuclear Corp. ^{14}C-Labeled methylaminehydrochloride (22.4 mCi/mmol) and ^3H_2O (5 mCi/ml) were purchased from The Radiochemical Centre (Amersham). Melibiose and TMG were obtained from Sigma Chemical Co. and *N,N'*-dicyclohexylcarbodiimide (DCCD) from Koch-Light Lab. Valinomycin was a gift of Eli Lilly and Comp. FCCP was a gift of Dr. P. Heytler.

Results and Discussion

To investigate whether a sodium gradient is sufficient to drive TMG uptake via TMG II in *S. typhimurium*, cells were depleted of energy by incubation with αMG plus azide as described by Koch [14] to prevent energization by oxidation of endogenous substrates or by glycolytic ATP. To prevent any residual metabolism KCN, iodoacetate and DCCD were added, while N_2 was passed through the cell suspension during incubations. Under these conditions, in cells induced for TMG II, addition of NaSCN results in the accumulation of TMG to a level several-fold above equilibration (Fig. 1). KSCN has no effect, as expected, when dealing with a Na^+-TMG symport system. One expects that the inward movement of Na^+ in symport with TMG has to be compensated electrically either by inward movement of a permeant anion or by efflux of a cation (for instance K^+ in the presence of valinomycin under in vitro conditions). Fig. 1 shows, however, that chloride, which is generally considered to be an impermeant anion in *E. coli* and *S. typhimurium*, is as effective as the permeant anion SCN^-. In contrast, in the case of galactose uptake driven by a H^+ gradient via galactose permease (a H^+-sugar symport system) the permeant anions SCN^- and NO_3^- are more effective than Cl^- in stimulating galactose transport [13]. The sodium salt of other "impermeant" anions such as phosphate, sulphate and citrate (all at 10 mM Na^+) also stimulated TMG uptake to the same extent as 10 mM NaSCN and Na^+-stimulated TMG uptake was not affected by the simultaneous presence of an externally directed potassium gradient plus valinomycin (not shown). In our search for ions which could compensate for the postulated influx of Na^+, we observed that proton extrusion is elicited by the addition of TMG to an anaerobic suspension of cells which had been starved for 1 h in medium A (Fig. 2, trace a, see also ref. 12). This proton extrusion is dependent on the presence of Na^+ and inhibited by FCCP and monensin. A similar pH change was reported by Tsuchiya et al. [14]. However, no H^+ movement could be detected in cells starved in the presence of αMG plus azide (Fig. 2, trace f). The two starvation methods used are probably

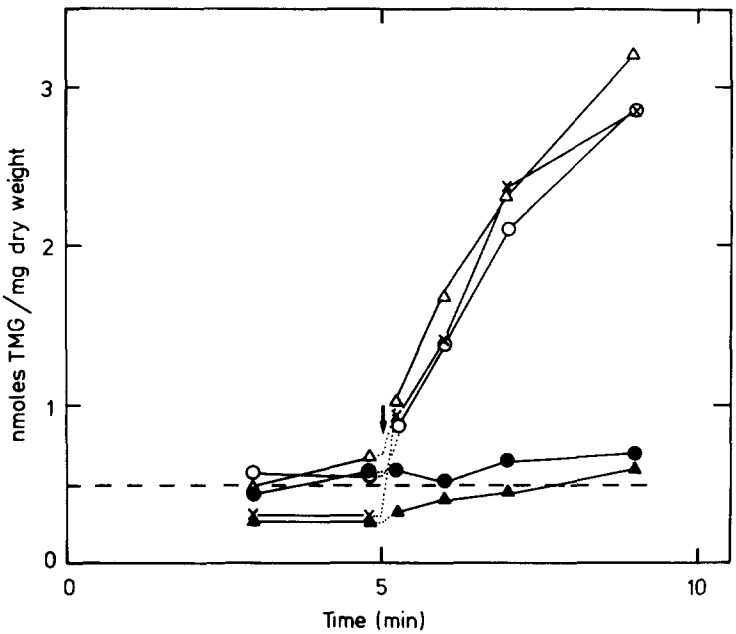

Fig. 1. Na^+-stimulated TMG uptake via TMG II in *S. typhimurium*. TMG transport was measured as described in ref. 16 using 0.2 mM [^{14}C]TMG (spec. act. 1835 cpm/nmol) in cells starved with αMG plus azide. At the arrow 10 mM of a sodium or potassium salt was added. X———X, NaSCN; ○———○, NaCl; ●———●, KSCN; ▲———▲, NaSCN, 5 μM FCCP; △———△, NaSCN, monensin 1 μg/ml. FCCP and monensin were present during preincubation (15 min). The dotted line represents equilibration.

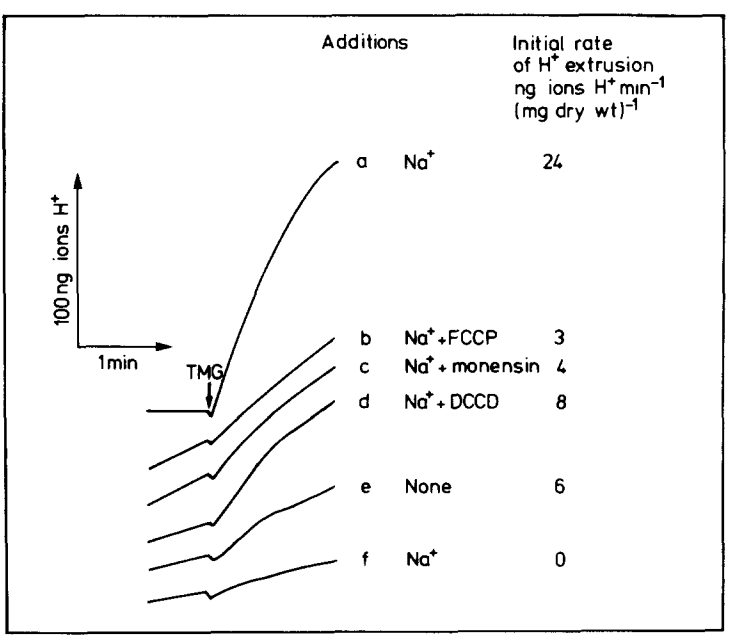

Fig. 2. pH Changes induced by TMG in *S. typhimurium*. a—e, cells were starved in medium A and pretreared with EDTA. Cells were preincubated for 30 min the presence of valinomycin (1 μg/ml). In a, b and c, [^{14}C]methylamine and ^3H_2O were present. Further additions: a, 10 mM NaCl; b, 10 mM NaCl and 5 μM FCCP; c, 10 mM NaCl and monensin (1 μg/ml); d, 10 mM NaCl and 200 μM DCCD; e, none. Cell density was 2.4 mg dry weight/ml. f is similar to a, except that cells were starved in the presence of αMG plus azide and labeled compounds were omitted. Cell density was 3.2 mg dry weight/ml. At the arrow 5 mM TMG was added. The initial rates were corrected for base-line drift.

not equally effective in depleting cells of endogenous energy. This might explain why sodium-stimulated TMG uptake is observed both in cells starved with αMG plus azide (Fig. 1) and in cells starved in medium A (not shown), but only the latter show proton extrusion. We have measured $\Delta\tilde{\mu}_{H^+}$ and ATP levels under the conditions of the experiments described above. Table I summarizes the values for $\Delta\psi$, ΔpH and $\Delta\tilde{\mu}_{H^+}$ obtained from the experiment shown in Fig. 2.

In cells starved in medium A without αMG and azide we find a $\Delta\tilde{\mu}_{H^+}$ of approximately 70 mV, consisting of a $\Delta\psi$ component of about 105 mV and a ΔpH component of about −35 mV. These values do not change significantly during the course of the experiment. 5 μM FCCP, which strongly inhibits H^+ extrusion, causes a decrease in $\Delta\psi$ and makes ΔpH even more negative, resulting in an almost complete collapse of $\Delta\tilde{\mu}_{H^+}$ to about 10 mV. Monensin also strongly decreases H^+ extrusion but has no effect on either $\Delta\psi$ or ΔpH (not shown). In cells starved in the presence of αMG plus azide under the conditions for uptake studies, $\Delta\tilde{\mu}_{H^+}$ remains rather constant at 30 mV during the course of the experiment, consisting of a $\Delta\psi$ of approximately 65 mV and a ΔpH component of about −35 mV (not shown). The high $\Delta\tilde{\mu}_{H^+}$ maintained throughout the experiment represented in Fig. 2 and Table I, indicates that starvation in the absence of αMG plus azide is not very effective and suggests that proton extrusion upon addition of TMG, is caused by metabolic activity. This activity is induced by the demand on the $\Delta\psi$ caused by Na^+ influx. Therefore, we measured the ATP concentration in the cells under the conditions described above. Table I shows that under conditions that proton extrusion occurs, the ATP level of the cells drops drastically immediately after addition of TMG. Another indication that ATPase activity is responsible for H^+ production is the inhibition by DCCD of H^+ extrusion (Fig. 2, trace d). In cells starved with αMG plus azide, ATP could not be detected.

It seems justified to conclude from these data that proton efflux is not directly coupled to Na^+-TMG symport, but is caused by metabolic activity via the ATPase which tries to maintain a constant $\Delta\tilde{\mu}_{H^+}$. The decrease in H^+ extru-

TABLE I

$\Delta\psi$, ΔpH, $\Delta\tilde{\mu}_{H^+}$ AND ATP CONCENTRATION IN *S. TYPHIMURIUM*

$\Delta\psi$, ΔpH, $\Delta\tilde{\mu}_{H^+}$ and ATP were measured as described in Materials and Methods. The values in experiments 1 and 2 were obtained from the experiment shown in traces a and b of Fig. 2. Intracellular water space was 57% of the total pellet water. Samples were taken before (t = 0 min) and after the addition of 5 mM TMG. Expt. 3 was a separate experiment in which the cell density was 4.3 mg dry weight/ml and labeled compounds were omitted.

Time (min)	Expt. 1 (no additions)			Expt. 2 (5 μM FCCP)			Expt. 3
	$\Delta\psi$	59 ΔpH	$\Delta\tilde{\mu}_{H^+}$	$\Delta\psi$	59 ΔpH	$\Delta\tilde{\mu}_{H^+}$	ATP
	(mV)			(mV)			(nmol/mg dry weight)
0	105	−38	67	88	−77	11	1.23
1 5 mM TNG added							
2	104	−35	69	83	−70	13	0.65
3.5	105	−33	72	84	−73	11	0.69
5	105	−33	72	83	−73	10	0.96
8	105	−33	72	78	−73	5	1.18

sion in the presence of monensin is in agreement with this interpretation, since, in this case, influx of Na^+ plus TMG will also result in metabolic activity to maintain $\Delta\tilde{\mu}_{H^+}$, but H^+ moving out will be immediately exchanged for Na^+ via monensin and, therefore, the pH change will be "buffered" away by the high Na^+ concentration. The inhibition by FCCP of TMG uptake is unexpected and may be caused by the fact that FCCP affects the permeability of the membrane for Na^+ [21] so that the Na^+ gradient is dissipated before it can drive TMG transport.

In cells starved in the presence of αMG plus azide, energy depletion is more effective, as shown by the low level of $\Delta\tilde{\mu}_{H^+}$ and ATP. In agreement with the above interpretation, no H^+ movement is observed in this case. However, these cells are still able to accumulate TMG in response to a Na^+ gradient. The observation that monensin has no effect on Na^+-stimulated TMG uptake may be explained by the rather large contribution of $\Delta\psi$ to the electrochemical Na^+ gradient ($\Delta\tilde{\mu}_{Na^+}$). Evidently, $\Delta\tilde{\mu}_{Na^+}$ is high enough to support the relatively slow uptake of TMG, even in the presence of monesin. The problem remains why, in extensively starved cells, Na^+-stimulated TMG uptake is independent of the anion present. Possibly, in cells starved in the presence of αMG plus azide, the passive permeability of the membrane for ions, even for the so-called impermeant anions, is high enough to compensate for the slower rate of Na^+-stimulated TMG influx. Previous studies [13] have shown that a limited H^+ influx, induced by galactose, is possible in a medium containing only chloride (Fig. 3A of ref. 13).

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