ß-GALACTOSIDE ACCUMULATION IN A Mg^{2+}-,Ca^{2+}-ACTIVATED ATPase DEFICIENT MUTANT OF E.COLI

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SUMMARY: A simple and rapid method for the isolation and identification of mutants of E.coli K12 lacking the Mg^{2+}-, Ca^{2+}-activated ATPase [E.C.3.6.1.3.] is described. The mutation is closely linked to ilv. The wildtype strain and the ATPase deficient mutant show comparable rates of TMG-accumulation. After inhibition of respiration TMG-accumulation is inhibited by 35% in the wildtype strain. In the mutant strain TMG-accumulation is abolished. It is concluded that the ATPase is necessary for the coupling of ATP-hydrolysis to the accumulation of TMG. Active TMG-accumulation is driven by either respiration or by ATP-hydrolysis via the ATPase.

ß-Galactosides are accumulated in E.coli by a genetically well defined system (1,2), though different models have been proposed to explain active ß-galactoside transport (2,3,4,5). It is unlikely that ß-galactosides are phosphorylated during the process of accumulation (3,4) as is the case for α-methylglucoside transport in E.coli (6). In membrane vesicles, accumulation of TMG is absolutely dependent on the presence of oxygen and of an oxidizable substrate, and is inhibited by cyanide or by uncoupling agents (7). Anaerobically grown cells of E.coli accumulate TMG under anaerobic conditions: this process is inhibited by uncouplers whereas the internal concentration of ATP, α-methylglucoside uptake and the ß-galactoside transport protein(s) are not affected (8). Recently, West and Mitchell (9) demonstrated a flow of protons into

Abbreviations used: AMS: mixture of 0.2% acetate, 0.2% malate and 0.2% succinate; ATPase: Mg^{2+}-,Ca^{2+}-activated ATPase [E.C. 3.6.1.3]; CCCP: carbonyl cyanide m-chlorphenylhydrazone; EGTA: ethyleneglycol-bis-(ß-aminoethylether)N,N'-tetraacetic-acid; ONPG: o-nitrophenyl-ß-D-galactoside; TMG: thiomethyl-ß-D-galactoside.

the cell of E.coli which is coupled to the inflow of ß-galactoside. This supports the idea that the accumulation of ß-galactosides is due to the operation of a proton-ß-galactoside symporter (10).

The question arises as to whether the ATPase is involved in the energy supply for ß-galactoside transport by ATP. This question can be answered with mutants lacking the ATPase recently described by Butlin, Cox, and Gibson (11). If the ATPase is involved in the energy supply by ATP the wildtype should accumulate TMG even after poisoning of the respiratory chain by cyanide; in the mutant strain, however, TMG-accumulation should be strongly dependent on respiration and, therefore, should be largely cyanide-sensitive.

Materials and Methods

Isolation of mutants deficient in Mg^{2+}-,Ca^{2+}-activated ATPase: Mutants of E.coli K12Ymel lac i^- were induced by mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine (12). After segregation in a glucose minimal medium, mutants, which can no longer grow in a minimal medium with AMS as carbon source were enriched for by penicillin selection. The surviving cells were plated on agar plates with glucose as sole carbon source and subsequently replica plated on agar plates containing AMS and on agar plates with glycerol as carbon source. Mutants were isolated which could no longer grow on AMS as sole carbon source but which could grow quite well on glycerol minimal medium.

Determination of Mg^{2+}-,Ca^{2+}-activated ATPase: Cells were grown in a minimal medium (13) containing 0.5% glucose and 0.2% casamino acids. After centrifugation the cells were washed twice with a buffer containing 100mM choline chloride, 2mM EDTA, 2mM Tris-HCl p_H 7.5. The cells were broken by ultrasonic disintegration and the ATPase was determined in this cell-free extract by measuring the change of hydrogen ion concentration at 37°C during the hydrolysis of ATP (14) in a

buffered system at p_H 7.5 containing 100mM choline chloride, 1mM EGTA, 2mM Tris-HCl, 3mM $MgCl_2$, 4mM ATP, and about 100µg/ml protein for wildtype extracts and 1mg/ml for extracts of mutant strains. The reaction was initiated by addition of ATP; then a defined amount of 0.1M KOH was added causing a small p_H change, and the time needed to neutralize this p_H change was determined. The p_H-change has been correlated with orthophosphate release by phosphate determination (15, unpublished work).
P1-transductions were performed as described by Lennox (16). Oxygen consumption was measured polarographically with a Clark-type oxygen electrode.

Results and Discussion

Whereas with AMS as sole carbon source, *E.coli* can only generate ATP by oxidative phosphorylation, glycerol is transformed after phosphorylation and oxidation to glyceraldehyde-3-phosphate from which ATP is generated by substrate level phosphorylation. Therefore mutants of *E.coli* deficient in ATPase can grow aerobically on glycerol. Approximately 30% of the mutants which do not grow on citric acid cycle substrates but grow on glycerol were deficient in ATPase activity. In six mutants deficient in ATPase activity it was shown by P1-transduction that the mutation is about 50% cotransducible with *ilv* (11). The apparent specific activity of the ATPase in the mutants (0.02-0.04µmole/min/mg) is 5-10% of that in the parental strain (Ø.4µmole/min/mg).

Strain A103c, used in the transport experiments, was constructed by transduction of the mutant ATPase allele from strain A103 into strain A1002 (K12Ymel *ilv*, *metE*, *lac i*) and selection for *ilv*$^+$ recombinants, of which 56% were deficient in ATPase activity.

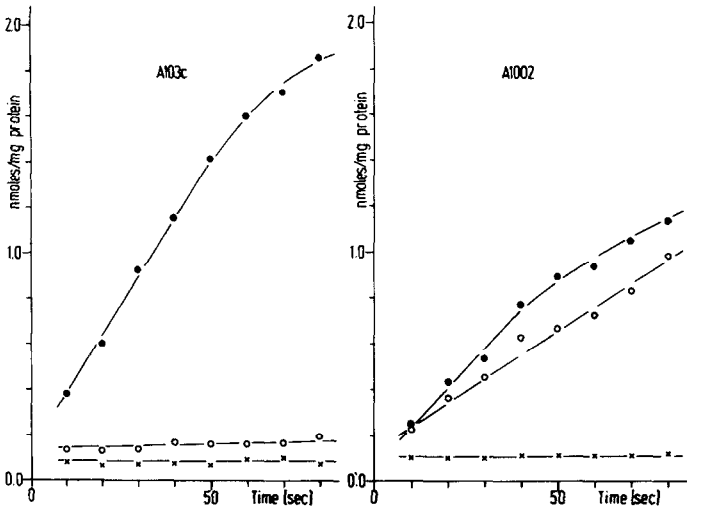

Figure 1: TMG-accumulation in A1002 and A103c

The cells were grown in Cohen-Rickenberg-(CR)-medium (13) containing 0.5% maltose and 0.01% of isoleucine, valine, and methionine to an optical density at 420nm of about 1. 0.45ml portions of this culture were incubated for 15min. with or without inhibitor at 25°C. Then, 0.05ml [^{14}C] TMG (10^{-4}M, specific activity 5C/mole) was added. The incubations were terminated by the addition of 5ml washing fluid at room temperature. The cells were immediately filtered on a membrane filter, covered with 5ml washing fluid and washed twice with 5ml washing fluid. The concentration of inhibitors were 2mM KCN and 50µM CCCP. Each point is an average of 4 (A103c) or 3 (A1002) measurements.

—•—•— no addition; —o—o— + KCN; —x—x— + CCCP.

Table 1: Initial rates of TMG-accumulation

The initial rates of TMG uptake which are taken from Fig.1 expressed in nmole/min/mg protein.

Addition / Strain	none	KCN	CCCP
A1002	0.94	0.62	< 0.05
A103c	1.52	< 0.05	< 0.05

Table 2: Respiration rates in A1002 and A103c

Cells were grown as described in the legend to Fig.1. Stimulation of respiration by maltose was measured at 37°C by addition of 20µl 20% maltose solution to 3ml CR-buffer in which whole cells (0.5mg protein) were suspended. NADH-oxidation was measured in the same way in broken cells (0.5mg protein) after the addition of 20µl 10% NADH solution. The KCN concentration was 2mM. The rates are expressed in ng-atom O/min/mg protein.
50µM CCCP did not influence oxygen consumption.
The inhibition by KCN was >95% after 2min.

Addition / Strain	Maltose	Maltose + KCN	NADH	NADH + KCN
A1002	530	< 20	780	< 20
A103c	700	< 20	1230	< 20

TMG-accumulation

Strains A1002 and A103c are constitutive for ß-galactosidase and lactose permease. In A1002 and A103c the specific activity of ß-galactosidase is 4.3 and 6µmole/min/mg respectively. Table 1 shows the initial rates and Figure 1 the kinetics of the TMG-accumulation in A1002 and A103c. The different rates of uptake in A1002 and A103c without inhibitors are probably due to different amounts of permease, which is in accordance with the specific activity of the ß-galactosidase.
If the cells are incubated at room temperature with KCN the rate of TMG-accumulation in A1002 is reduced by 35%; under these conditions the rate of TMG-accumulation in A103c is abolished. This result is in accordance with the observation that in mutants deficient in ATPase the energy linked transhydrogenase reaction can be driven only by respiration and not by ATP (17,18). TMG-uptake is entirely inhibited in A1002 and A103c by CCCP. Respiration with maltose as substrate

Table 3: In vivo ONPG-hydrolysis in A1002 and A103c

In vivo ONPG-hydrolysis was measured spectrophotometrically at 25°C at 420nm. The rate is expressed in nmole/min/mg protein. Sample cuvette: CR-buffer, 2mM ONPG whole cells corresponding to 0.02-0.03 mg protein/ml and 2mM KCN or 50 µM CCCP. Reference cuvette: CR-buffer, 2mM ONPG, 3.3mM TDG, whole cells corresponding to 0.02-0.03 mg protein/ml and 2mM KCN or 50µM CCCP.

Addition / Strain	none	KCN	CCCP
A1002	270	141	96
A103c	210	133	85

in whole cells or with NADH in cell-free extracts is completely inhibited (≥95%) by KCN (Table 2) but CCCP shows no effect. The specific activity of respiration in A103c is higher than in strain A1002. Butlin et al. (11) assume that the higher rate of respiration is due to uncoupling in the mutant.

In contrast to TMG-accumulation, the in vivo ONPG hydrolysis (1,19), which is a measure of functional ß-galactoside permease (20) is inhibited by cyanide in both strains to the same extent (Table 3). CCCP reduces the rate to 1/3 of the uninhibited rate in both cases. This suggests that in A103c the ß-galactoside permease protein itself is not more sensitive to KCN than in A1002.

The experiments of West and Mitchell (9) support the idea that the accumulation of ß-galactosides is due to the operation of a proton-ß-galactoside symporter (10), which implies that a proton gradient (external acid) must be established across the coupling membrane for the active uptake of TMG. Strain A1002 has a functional ATPase and is able to accumulate TMG after

respiratory inhibition by cyanide. In strain A103c, on the other hand, which lacks ATPase activity, the uptake of TMG is completely inhibited by cyanide. The active accumulation of TMG in both strains is totally inhibited by the uncoupling agent CCCP which catalyzed rapid proton equilibration across the membrane (10,21). The results reported here are in agreement with the proposal that a proton gradient must be established across the membrane for active TMG-accumulation in E.coli and that this gradient can be generated either by respiration or by the hydrolysis of ATP via the Mg^{2+}-,Ca^{2+}-dependent ATPase (10,21).

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