Artificially Induced Active Transport of Amino Acid Driven by the Efflux of a Sugar via a Heterologous Transport System in De-energized *Escherichia coli*

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Consistent with the model of an H^+ cotransport, amino acid uptake can be driven by a proton gradient generated by an efflux of sugar when the normal energy sources are suppressed. Heterologous countertransport is completely inhibited by uncouplers unlike homologous countertransport. Positive coupling was obtained with methyl thiogalactoside/proline, methyl thiogalactoside/phenylalanine, gluconate/proline; however, the poor coupling efficiency suggests a more complex sequence of reactions.

The energy source for the uphill transport of several solutes by a variety of transport systems in *Escherichia coli* exhibits the properties of an electrochemical gradient of H^+ ions and the mechanism of its utilization is consistent with a model of H^+ /solute cotransport (Berger & Heppel, 1974; Harold, 1977).

The first statement implies that the membrane can be energized by the respiratory chain or by the membrane-bound ATP synthetase functioning as an adenosine triphosphatase and that artificially generated transmembrane potential (a K^+ diffusion potential induced by valinomycin) or an artificially generated pH difference (inside alkaline) can induce a transient uphill transport when the normal energy sources have been suppressed (Altendorf *et al.*, 1975; Schuldiner & Kaback, 1975; Lagarde, 1977; Flagg & Wilson, 1977).

The second statement is supported by the observation of H^+ ion fluxes accompanying downhill (West & Mitchell, 1973) or uphill (Robin & Kepes, 1973; Lagarde, 1977) movements of the solutes via their specific carriers.

It was also shown, that the downhill movement of one solute can drive uphill in the opposite direction another solute, substrate of the same transport system (Winkler & Wilson, 1966; Kepes, 1969; Bentaboulet & Kepes, 1977), in conformity with the predictions of the mobile-carrier model and that this homologous countertransport could be directed to either side of the membrane; the reversal of the driving flux causes the uphill driven flux to reverse (Teather *et al.*, 1977). This countertransport is specific and depends on the integrity of a functional carrier (Kepes, 1969; Wong & Wilson, 1970), but its detection depends on the elimination of the energized state of the membrane, otherwise the phenomenon is overshadowed by the

active transport. If a downhill flux of substrate is coupled to a flux of H^+ ions, it should be able to establish a transient electrochemical gradient of H^+ at the expense of the dissipation of the initial chemical gradient of the solute, and this transient H^+ gradient could be used for the uphill transport of another solute via a different carrier, provided that it also works according to the model of H^+ symport. Among possible solutes, several amino acids and sugars can be listed (Berger, 1973; Berger & Heppel, 1974; Robin & Kepes, 1973; West & Mitchell, 1973). The detection of such heterologous countertransport processes is the objective of the present work. Positive results were obtained with the following solute pairs: methyl thiogalactoside/proline, methyl thiogalactoside/phenylalanine, gluconate/proline.

Materials and Methods

Bacteria

Strains used were ML308 and AR13, a gluconate kinase-deficient strain isolated in this laboratory (Robin & Kepes, 1975) from strain DF 1070 (Fraenkel, 1968). Cells were grown in minimal medium supplemented with 0.4% glycerol and 0.5 mM-gluconate in the case of strain AR13 to induce the gluconate-transport system.

Cells were harvested by centrifugation or filtration, washed once with medium 63 (Rickenberg *et al.*, 1956) and once more with cold water at 4°C for eliminating the internal pool of amino acids and of gluconate.

Cells were usually resuspended in medium 63 containing 50 µg of chloramphenicol/ml.

Countertransport

Washed bacteria were preloaded in concentrated suspension (25–50 mg dry weight/ml) in medium 63 containing 5 mM-KN₃/2 mM-KCN, by incubation with 100 mM-methyl thiogalactoside or 20 mM-gluconate for 45 min at room temperature.

At the start of the experiment cells were diluted 200 times in the same medium, so as to establish an outward-directed gradient of the preloaded substrate. The dilution medium still contained the energy inhibitors together with the radioactive solute to be driven uphill.

The concentration of the driven substrate was kept lower than 2 K_i so that significant gradients could be built up with a moderate total flux.

Samples (1 ml) were withdrawn, filtered, washed with medium 63 and counted for radioactivity with liquid scintillator.

Control experiments were performed with non-preloaded bacteria; the preloading substrate was then added to the dilution medium so that the composition of control was identical with the experimental sample.

Another procedure could also be used instead of dilution: preloaded bacteria were filtered and resuspended in the exit medium as described above.

Chemicals

Carbonyl cyanide *m*-chlorophenylhydrazone was from Calbiochem, La Jolla, CA, USA.; [¹⁴C]methyl

β -D-thiogalactopyranoside (35 mCi/mmol) and [¹⁴C]-proline (250 mCi/mmol) were from CEA, Saclay, France; [¹⁴C]gluconic acid (sodium salt; 3.9 mCi/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K.

Results

Proline accumulation in response to a methyl thiogalactoside gradient

In a preliminary experiment, we ensured that the proline transport was inhibited by 90% by 5 mM-KN₃ and 2 mM-KCN (Table 1).

Bacteria were then concentrated in the presence of N₃⁻ and CN⁻ and preloaded with 100 mM-methyl thiogalactoside. The inset of Fig. 1 shows that upon dilution, the methyl thiogalactoside efflux can drive an uphill transport of proline, which is not observed with unloaded bacteria. Phenylalanine, whose transport is also N₃⁻/CN⁻-sensitive, gives a similar result in response to the outflux of methyl thiogalactoside. The proline concentration in the bacteria reaches the equilibrium value in a few seconds and keeps increasing for 1.5–2 min to reach a peak at 5–10-fold accumulation. During the following minutes the concentration drops to a value similar to that of the non-preloaded control and not very different from the concentration in the medium. From the second half of the curve an approximate time constant can be derived. It is not very different from the time constant

Table 1. *Comparison of heterologous and homologous countertransports*

Washed cells (10 mg dry weight/ml) were suspended in medium 63 containing 5 mM-KN₃, 2 mM-KCN and the preloading substrate at 20°C. Then 90 min later, 0.15 ml of preloaded cells were filtered and resuspended in 30 ml of medium containing (except for last row, which describes uninhibited transport) 5 mM-KN₃, 2 mM-KCN and either μ M-[¹⁴C]-proline, 0.5 mM-[¹⁴C]methyl thiogalactoside or 0.1 mM-[¹⁴C]gluconate. Experiments were made with doubly induced strain AR13, except when marked (*) where strain ML308 was used.

Preload (mM)	Proline uptake at pH 7.8 (μ mol/g)		Methyl thiogalactoside uptake at pH 7.4 (μ mol/g)		Gluconate uptake at pH 7.4 (μ mol/g)	
	2 min	10 min	2 min	10 min	2 min	10 min
(Methyl thiogalactoside)						
100	0.28	0.08*	33	8	3	3
50	0.20	0.08*				
20	0.14	0.08*				
10	0.11	0.08*				
5	0.08	0.08*				
0	0.08	0.08*	8	8	6	6
Gluconate						
20	0.21	0.05	4	4	37	5
0	0.05	0.05	8	8	5	5
Proline						
0.2	0.16	0.04*				
0	0.04	0.04*				
Active transport (no preload, no energy inhibitors)		15		80		200

Fig. 1. Methyl thiogalactoside/proline countertransport: effect of carbonyl cyanide chlorophenylhydrazone

ML308 cells were inhibited with 5mM-KN₃ and 2mM-KCN, preloaded with 100mM-methyl thiogalactoside; at zero time, they were diluted 200 times in medium 63 containing the same inhibitors, 0.5mM-methyl thiogalactoside, 1 μM-[¹⁴C]proline (70mCi/mmol) and the indicated concentrations of uncoupler (▲). Control experiments were with non-preloaded bacteria (○). The values of [Proline]_{in} represent the internal concentration reached 2min after dilution. The inset shows an example of a countertransport experiment (no uncoupler in the dilution medium); [¹⁴C]proline uptake in methyl thiogalactoside-preloaded (●) and non-preloaded (○) bacteria.

Fig. 2. pH-dependence of the methyl thiogalactoside/proline countertransport

ML308 cells were washed with medium 63 adjusted to pH values ranging from 5.5 to 7.95; countertransport experiments were performed with 5mM-KN₃ and 2mM-KCN, 100mM-methyl thiogalactoside preloading, 1 μM-[¹⁴C]proline or 0.5mM-[¹⁴C]methyl thiogalactoside in the dilution medium. Methyl thiogalactoside/proline countertransport (●) and methyl thiogalactoside/methyl thiogalactoside countertransport (▲) peak at different pH values. ◆, Maximal proline active transport measured with uninhibited cells and 1 μM-[¹⁴C]proline.

of the driving flux, the exit of methyl thiogalactoside (Bentaboulet & Kepes, 1977).

Effect of carbonyl cyanide chlorophenylhydrazone on the heterologous countertransport

When 1–10 μM-carbonyl cyanide chlorophenylhydrazone are added to the dilution medium in an experiment identical with the one already described, the maximal transient accumulation of proline in methyl thiogalactoside-preloaded bacteria is considerably decreased and even abolished (Fig. 1).

pH-dependence of the methyl thiogalactoside/proline countertransport

Methyl thiogalactoside/proline countertransport measured by the peak value of the transient influx, is more effective at alkaline pH than at acid pH (see Fig. 2).

This parallels the pH-dependence of the proline accumulation in normally aerated bacteria or in N₃⁻/CN⁻-pretreated bacteria (inhibitors being removed at the time of the accumulation) which

exhibits maximal initial velocities and a maximal concentration factor at alkaline pH (Fig. 2). In contrast, the efficiency of homologous methyl thiogalactoside/methyl thiogalactoside countertransport increases and approaches one-to-one when the pH of the medium is decreased from 8 to 6, although the methyl thiogalactoside efflux, which is the driving force, is substantially unchanged (Bentaboulet & Kepes, 1977).

Coupling efficiency in relation to the methyl thiogalactoside gradient

When decreasing the methyl thiogalactoside gradient, by decreasing the methyl thiogalactoside concentration during preloading, the proline accumulation is lowered (see Table 1). Half-maximal proline overshoot can be reached by the efflux of approx. 20mM-methyl thiogalactoside. Table 1 also indicates

that the inward flux of proline uses only a very small fraction of the potential gradient caused by the efflux of methyl thiogalactoside whatever this potential gradient is.

Comparatively the homologous proline/proline countertransport exhibits a coupling efficiency 250 times better than that of methyl thiogalactoside/proline.

Heterologous gluconate/proline countertransport

De-energized cells preloaded with gluconate can achieve a transient uptake of proline that is about the same as measured with methyl thiogalactoside preload (refer to Table 1).

Discussion

The experiments reported here show that two opposite fluxes mediated by two distinct transport systems can be coupled in de-energized cells, suggesting the existence of a common intermediate, which allows the use of one substrate flux for the accumulation of another substrate against a concentration gradient.

The common co-substrate of the two transport systems coupled this way is most likely the H^+ ion. This hypothesis is strengthened by the sensitivity of the coupling to the proton-conducting agent carbonyl cyanide chlorophenylhydrazine.

The possibility of a coincidental coupling restricted to a given pair of systems in a single bacterial strain has been ruled out by driving a transient uphill flux of phenylalanine as well as of proline by an efflux of methyl thiogalactoside via the *lac* permease, as well as by driving a proline uptake in the same way by a downhill efflux of gluconate via the gluconate permease, and carrying out experiments with two different bacterial strains.

The methyl thiogalactoside/proline countertransport exhibits an increased efficiency at the more alkaline pH values. This is parallel to the pH-dependence of active transport of proline and might suggest that the proline carrier is rate limiting in the reaction sequence. But the pH-dependence is opposite to that observed in the homologous methyl thiogalactoside/methyl thiogalactoside countertransport and, if this is taken as a measure of the potency of the lactose carrier that supports the driving flux, it could be considered as paradoxical. In contrast one could argue that at pH 8 external methyl thiogalactoside stops using the energy generated by the flux of preloaded methyl thiogalactoside, thus having little competitive effect on proline uptake. Assuming a stoichiometry of one or two in the methyl thiogalactoside/ H^+ cotransport and in the H^+ /proline cotransport (Ramos & Kaback, 1977), one would expect that the driving flux and the

driven flux are of the same order of magnitude. This could of course be limited by the carrying capacity of either of the two transport systems. In fact in the present experiments the lactose carrier or the gluconate carrier work close to their V_{max} , i.e. about $100 \mu\text{mol/min per g}$, whereas the maximal proline fluxes in active transport amount to only $2\text{--}4 \mu\text{mol/min per g}$. It remains nevertheless a gap to explain why the proline flux carried by the heterologous countertransport is only of the order of $0.2 \mu\text{mol/min per g}$.

At each step of the sequence a leak is possible. Part of the methyl thiogalactoside or gluconate can leak out of the cell by-passing their transport systems and escape the obligation of being accompanied by an H^+ ion. The H^+ ion gradient generated in this step can be dissipated in a variety of energy-requiring processes, notably other transport systems than the two participating in the sequence, and even in ATP synthesis (Maloney *et al.*, 1974). Part of the H^+ gradient can also be dissipated by a non-specific leak.

Finally a leak of proline during the countertransport can contribute to diminish the observed coupling efficiency. The coupling efficiency remains poor if maximal gradients are compared instead of fluxes. The initial gradient of the driving solute is routinely 200-fold, whereas the peak gradient of proline is only 7-fold.

Similar discrepancies are common in experiments reported in the literature where a valinomycin-induced K^+ gradient is the driving flux or an acid pulse in the medium, although in the latter case only gradients, but not fluxes, can be compared.

The failure to demonstrate heterologous countertransport in the following solute pairs (driving solute first): proline/methyl thiogalactoside, proline/gluconate, gluconate/methyl thiogalactoside, methyl thiogalactoside/gluconate might be just another aspect of the poor-coupling-high-leak phenomenon. Its effect could be reinforced if the H^+ gradient became operative only above some threshold. But in view of these positive but poor coupling experiments the hypothesis of a more complex sequence of reactions, with more steps and therefore a larger number of leak pathways remains open.

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