Absence of a unique relationship between active transport of lactose and protonmotive force in *E. coli*

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The relationship between active transport of lactose via the lactose permease and the protonmotive force has been determined in *E. coli* cells using either the respiratory chain inhibitor cyanide or protonophores to decrease the protonmotive force progressively. In contradiction with the prediction of the delocalized chemiosmotic theory, two different relationships were obtained depending on the method used.

Active transport *Lactose permease* *Protonmotive force* *Chemiosmotic theory* *Flow-force relationship* (E. coli)

1. INTRODUCTION

There is widespread agreement that the problem of energy transduction in mitochondria, chloroplasts and bacteria has been solved in terms of proton coupling as proposed by the chemiosmotic theory [1]. Nevertheless, a certain number of reports seem to contradict specific quantitative predictions of the original delocalized chemiosmotic theory, thus leading several authors to propose modifications of that theory (reviews [2,3]). One of the most compelling predictions of the theory is that the relationship between flux and force should be unique and not depend on how the latter is varied. Yet, in mitochondria, the rate of respiration is a steeper function of the protonmotive force (Δp) when the former is stimulated by ADP than when it is stimulated by protonophores [4,5]; also, several reports indicate that in mitochondria [6–8] and chromatophores from photosynthetic bacteria [9], the rate of ATP synthesis is a different function of Δp, depending on whether Δp is decreased by protonophores or electron-transport inhibitors. In contrast, a unique relationship between ATP synthesis and protonmotive force has been reported for submitochondrial particles [10]. In thylakoids, unique [11] and non-unique [12] relationships have been reported. These discrepancies can be attributed to the variety of the systems investigated and/or to the difficulties involved in the measurement of the different parameters (rate of phosphorylation, $\Delta\psi$, ΔpH).

We chose to investigate this problem with reference to secondary active transport. Indeed, the rate of solute transport or solute accumulation is easily monitored as compared to the rate of phosphorylation. The system used, the lactose permease of *E. coli*, may be considered as the archetype of proton/solute cotransport. The lac permease of *E. coli*, an intrinsic membrane protein of M_r 46 500 coded by the *lac y* gene, has been demonstrated to catalyse cotransport of β-galactosides with protons. The permease has been

Abbreviations: TPP, tetraphenylphosphonium ion; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TCS, 3,3′,4′,5-tetrachlorosalicylanilide; PCMBS, *p*-chloromercuribenzenesulfonate; Δp, protonmotive force; ΔpH, transmembrane pH difference; $\Delta\psi$, transmembrane electrical potential difference; Δp_{lac}, $(RT/F) \cdot \ln([\text{lactose}]_{in}/[\text{lactose}]_{out})$

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purified, reconstituted in proteoliposomes and shown to catalyse active transport upon imposition of artificial ion gradient (review [13]).

Here, the protonmotive force was progressively diminished in whole cells by controlled addition of either protonophores (permeabilization of the membrane to protons) or cyanide (inhibition of the respiratory chain); in each case Δp and the rate of lactose transport or lactose accumulation were measured in parallel. We show that there is no unique relationship between active transport of lactose and the protonmotive force. We discuss these results in connection with those of Elferink et al. [14] who recently proposed the existence of a direct link between the lactose permease and the respiratory chain.

2. MATERIALS AND METHODS

Cells of *E. coli* ML 308225 (i^-, z^-, y^+, a^+) were grown in minimal medium M9 containing 0.4% glycerol as the sole carbon source. To render the membrane permeable to the membrane potential probe, the cells were treated with EDTA according to [15]. When the cells were treated with valinomycin, the procedure of Bakker was used [15]: valinomycin (10 nmol/mg dry wt cells) was added during EDTA treatment. All assays were performed at 25°C in 10 mM Tris-HCl, 150 mM NaCl (pH 7.65) or in 150 mM sodium phosphate buffer (pH 7.65), in the presence of 0.4% glycerol. Except for the cytoplasmic volume determination, the cell concentration was 1 mg dry wt/ml.

The cytoplasmic volume was determined with ^3H_2O (0.11 MBq/ml) and [^{14}C]sucrose (0.3 μM, 20 GBq/mmol), according to [16]. 1.5 ml cells (5 mg dry wt/ml) were incubated for 5 min in the presence of the radioactive probes, catalase (5000 U/ml) and 10 μl of 0.2 M H_2O_2 to prevent anaerobiosis. After centrifugation, an aliquot (100 μl) of the supernatant and the pellet were counted for radioactivity on the preset $^3H/^{14}C$ program of a liquid scintillation counter.

ΔpH was estimated from the accumulation of [^{14}C]benzoate (10 μM, 1.97 MBq/mmol) in the presence of tritiated water (0.11 MBq/ml), incubated for 5 min in the presence of the cells [16]. The method of separation was centrifugation.

$\Delta\psi$ was estimated from the accumulation of [^{3}H]TPP (10 μM final concentration, 10 GBq/mmol) or [^{14}C]TPP (10 μM final concentration, 2.17 GBq/mmol) incubated for at least 10 min in the presence of the cells [16]. The method of separation was centrifugation or filtration [20]. TPP uptake was corrected for unspecific binding by subtracting a blank obtained under identical conditions except that the cells were pretreated with protonophores.

The rate of lactose transport was estimated from the accumulation of lactose (500 μM final concentration, either tritiated, 0.37 GBq/mmol or ^{14}C-labelled, 5.5 MBq/mmol) at 20 s, using filtration [20]. When $\Delta\psi$ was measured in parallel, [^{14}C]- or [^{3}H]TPP was added 10 min before the addition of lactose.

Lactose accumulation at the steady state was determined from the accumulation of [^{14}C]lactose (10 μM, final concentration, 0.63 GBq/mmol) after 30 min incubation. When $\Delta\psi$ was measured in parallel, [^{3}H]TPP was added at the same time. Other additions were as indicated. Cells were separated from the medium by centrifugation or filtration. In both cases (rate of lactose transport and lactose accumulation at the steady state) lactose uptake was corrected by subtracting a blank performed on cells pretreated with 300 μM PCMBS, a thiol reagent which blocks the permease.

Oxygen consumption was determined polarographically, using a Clark oxygen electrode.

[^{3}H]Lactose, [^{3}H]TPP, [^{14}C]sucrose, [^{14}C]benzoate and tritiated water were from CEA, France. [^{14}C]Lactose and [^{14}C]TPP were from Amersham, England. TCS was a generous gift from Dr I.R. Booth (Aberdeen). All other materials were of reagent grade and obtained from commercial sources.

3. RESULTS AND DISCUSSION

3.1. *Determination of the protonmotive force*

The cytoplasmic volume is a significant parameter in the measurement of protonmotive force and internal lactose concentration. Measurements of the cytoplasmic volume, using sucrose and tritiated water, yielded values between 0.9 and 1.1 μl/mg dry wt. When bacteria were treated with protonophores (10 μM TCS) or cyanide (2 mM) no significant change in the cytoplasmic volume was detected; when bacteria were treated with valino-

mycin in the presence of 150 mM external potassium, the internal volume was 1.5 μl/mg dry wt. All the calculations ($\Delta\psi$, Δp_{lac}) were performed using the experimentally determined internal volume.

To reduce Δp to its electrical component, all the experiments were performed at pH 7.65 as it has been shown that the internal pH of *E. coli* is buffered around 7.6 ($\Delta pH = 0$) [17]. Experiments with benzoate as a ΔpH probe gave a residual uptake corresponding to a ΔpH of less than 0.1 which was probably due to unspecific binding since it was uncoupler insensitive. At pH 7.65, bacteria treated with 10 μM TCS or CCCP, or with 2 mM cyanide or with valinomycin in the presence of potassium showed no difference in benzoate accumulation as compared to untreated bacteria. We may thus be reasonably confident that under all these conditions, $\Delta\psi = \Delta p$.

The $\Delta\psi$ probe used here was TPP. This poses the problem of the correction for the unspecific binding of this probe, particularly important in whole cells. In this study, TPP accumulation was corrected for TPP binding obtained in the presence of high concentration of uncouplers [19]. Other procedures of correction have been proposed [18]. However, one may expect, whatever the calculated value, a 1:1 relation between the actual and calculated $\Delta\psi$ value which is the point of importance in this study.

It should be stressed that due to the high unspecific binding and indepedently of the method of correction, the accuracy in the measurement of $\Delta\psi$ decreases rapidly with decreasing $\Delta\psi$. Thus, for example, 40 mV corresponds to an accumulation of the free probe which is only 10% of the binding. In view of the experimental dispersion it is impossible to give a precise determination of $\Delta\psi$ below 50–60 mV.

3.2. *Non-unique relationship between active transport and protonmotive force*

Fig.1 shows the variation of the initial rate of lactose transport with $\Delta\psi$ for an external lactose concentration of 0.5 mM, corresponding to the K_T of transport [20]. Controlled additions of the protonophore TCS (from 0.3 μM to 10 μM), which permeabilizes the membrane to protons, result in a progressive decrease of $\Delta\psi$ and in a concomitant non-linear decrease of the rate of lactose transport. Controlled additions of cyanide (up to 2 mM),

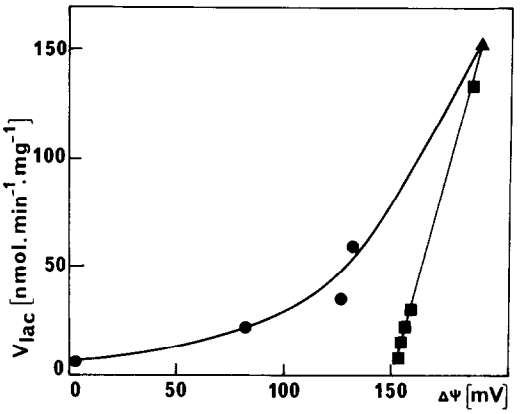

Fig.1. Rate of lactose transport as a function of $\Delta\psi$. Without addition (▲); in the presence of increasing concentration of TCS, from 0.3 to 10 μM (●); in the presence of increasing concentration of cyanide, from 0.2 to 2 mM (■). In the latter case, cells were preincubated with cyanide for 20 min.

which progressively inhibits respiration, from 25 nmol O_2/mg dry wt per min to 0 result in a decrease of $\Delta\psi$ from 180–190 to 150–120 mV. In the latter case, the variation of the rate of lactose transport with $\Delta\psi$ is much steeper than when $\Delta\psi$ is varied with the uncoupler. In a series of five different experiments the rate of lactose transport

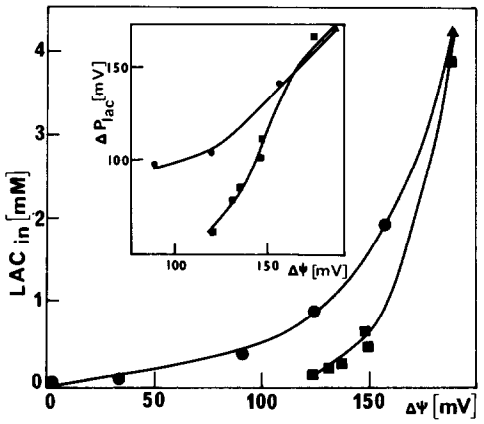

Fig.2. Steady-state accumulation of lactose as a function of $\Delta\psi$. Without addition (▲); in the presence of TCS, from 0.3 to 10 μM (●); in the presence of cyanide, from 0.2 to 2 mM (■). Inset: Δp_{lac} as function of $\Delta\psi$, calculated from the same data.

when respiration was completely blocked ($\Delta\psi$ = 120–150 mV), was 3–5-times lower than in the presence of uncoupler for the same value of $\Delta\psi$.

A similar pattern was observed when the steady-state level of accumulation of lactose was determined and plotted against $\Delta\psi$. In this case the external lactose concentration was low (10 μM) in order to maximize accumulation. The results obtained by the filtration method are shown in fig.2. Under conditions where the respiratory chain was blocked (2 mM cyanide $\Delta\psi$ = 120 mV), the accumulation ratio of lactose was 10 (Δp_{lac} = 60 mV), while upon titration with the protonophore and for the same $\Delta\psi$ value (120 mV), the accumulation ratio of lactose was still 60 (Δp_{lac} = 105 mV). Experiments using the centrifugation technique instead of filtration gave the same results. Also, the same pattern was observed when CCCP was used instead of TCS (not shown).

It should be emphasized that, in both cases (figs 1,2), the large difference in the rate of transport or in the accumulation of the solute between non-respiring and partially uncoupled cells occurs in a $\Delta\psi$ region (120–150 mV) where the determination of TPP accumulation is precise, as discussed above.

The low level of lactose transport observed in the presence of cyanide cannot be due to an unspecific effect of this compound on the permease since cyanide was shown to have no effect on lactose transport, for the concentrations used in this study, in a mutant devoid of a respiratory chain [21].

3.3. *Is there a direct link (not involving protons) between active transport and respiration?*

Elferink et al. [22] reported that cyanide considerably inhibits lactose transport in *E. coli* cells with little action on $\Delta\psi$, as confirmed by our data. While this study was in progress the same group reported that complete collapse of $\Delta\psi$ by valinomycin in the presence of potassium or by gramicidin, resulted in little or no effect on the rate of lactose transport and accumulation [14]. This led the authors to propose that both a Δp and electron transfer can energize lactose transport [14]. Under anaerobic conditions, the lactose permease would function purely as a proton symport. Under aerobic conditions, extra energy would be provided by a direct (not involving proton) link with the respiratory chain, which would account for the large remaining solute accumulation at zero Δp.

The experimental results of Elferink et al. [14] are in contradiction with the large body of evidence reported in the literature showing that in *E. coli* cells, collapse of the protonmotive force by protonophores supresses lactose accumulation [23]. In our study, under conditions identical to those of Elferink et al. (i.e. *E. coli* cells provided with external energy source), we found that addition of the protonophores TCS (10 μM), or CCCP (10 μM), or 2,4-dinitrophenol (2 mM) resulted in a decrease in the accumulation ratio of lactose from some 500-fold to 1–2 as measured by the centrifugation method. A possible effect of respiration cannot be invoked here: addition of those uncouplers has little or no effect on respiration (not shown). The classical uncoupler CCCP was not tested by Elferink et al. [14]; we therefore investigated whether their result could be due to their mode of uncoupling by valinomycin-potassium. We observed that, in valinomycin-treated cells, as the potassium external concentration was raised from 0 to 150 mM, TPP uptake decreased from a value corresponding to a $\Delta\psi$ = 165 mV to the value observed in the presence of high concentration of protonophores. The lactose accumulation ratio decreased in parallel with increasing concentration of potassium from 500 (Δp_{lac} = 160 mV) to 7 (Δp_{lac} = 50 mV). No ΔpH under these conditions was detected from benzoate accumulation, and nigericin (7 μM) had no effect on this residual accumulation of lactose.

These results are in good agreement with those of Ahmed and Booth [24] who reported a remaining 40 mV lactose accumulation under the same conditions in cells using endogenous energy sources. These results are clearly different from those of Elferink et al. [14]: while they report that complete depolarization decreases the accumulation ratio of lactose by a factor of 3 (Δp_{lac} decreasing from ~ 150 to ~ 120 mV), in our case, the remaining lactose accumulation ratio was lower by a factor of 70 as compared to its value when the cells were incubated in the absence of potassium. The residual Δp_{lac} of 50 mV that we observe can obviously still be accounted for by a chemiosmotic mechanism, since the presence of a remaining $\Delta\psi$ of a sufficient magnitude cannot be ruled out.

4. CONCLUSION

It has been demonstrated, in whole cells as well as in reconstituted systems, that the lac permease is a proton symport [13]. We confirm here that, in whole cells, protonophores completely collapse lactose accumulation. Depolarization by potassium in the presence of valinomycin reduces lactose accumulation to 1–2% of its original level, corresponding to a remaining Δp_{lac} which could be largely accounted for by a residual $\Delta\psi$, difficult to determine in whole cells. In consequence, we think that the proposal by Elferink et al. [14] of a direct link (not involving protons) between the respiratory chain and the lac permease, operating in addition of the protonmotive force, is not justified.

Nevertheless, the results reported here, showing that the rate of lactose transport through the lactose permease, as well as lactose accumulation, is different for the same value of the protonmotive force, depending on whether this parameter is varied by permeabilization of the membrane to protons or by inhibition of the respiratory chain, clearly contradict the classical delocalized chemiosmotic theory. This observation thus extends to the field of transport the problems arising from similar discrepancies originally reported for various phosphorylating systems. To account for them a certain number of modifications of the theory, implicating localized or partially localized protons, have been proposed [2,25]. The experiments reported here cannot discriminate between these proposals. Nevertheless, secondary active transport systems should prove to be simple and helpful tools in the investigation of these problems.

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REFERENCES

[1] Mitchell, P. (1979) Eur. J. Biochem. 95, 1–20.
[2] Westerhoff, H.V., Melandri, B.A., Venturoli, G., Azzone, G.F. and Kell, D.B. (1984) Biochim. Biophys. Acta 768, 257–292.
[3] Ferguson, S.J. (1985) Biochim. Biophys. Acta 811, 47–95.
[4] Padan, E. and Rottenberg, H. (1973) Eur. J. Biochem. 40, 431–437.
[5] Azzone, G.F., Pozzan, T., Massari, S. and Bragadin, M. (1978) Biochim. Biophys. Acta 501, 296–306.
[6] Zoratti, M., Pietrobon, D. and Azzone, J.F. (1982) Eur. J. Biochem. 126, 433–451.
[7] Mandolino, G., De Dantis, A. and Melandri, B.A. (1983) Biochim. Biophys. Acta 723, 728–439.
[8] Zoratti, M. and Petronilli, V. (1985) FEBS Lett. 193, 276–282.
[9] Casadio, M., Baccarini-Melandri, A. and Melandri, B.A. (1978) FEBS Lett. 87, 323–328.
[10] Sorgato, C.N., Lippe, G., Seren, S. and Ferguson, S.J. (1985) FEBS Lett. 181, 323–327.
[11] Portis, A.R. and McCarty, R.E. (1974) J. Biol. Chem. 249, 6250–6254.
[12] Sigalat, C., Haraux, F., De Kouchkovsky, F., Nhu Hung, S.P. and De Kouchkovsky, Y. (1985) Biochim. Biophys. Acta 809, 403–413.
[13] Kaback, H.R. (1983) J. Membrane Biol. 76, 95–112.
[14] Elferink, M.G.L., Hellingwerf, K.J. and Konings, W.N. (1985) Eur. J. Biochem. 153, 161–165.
[15] Bakker, E.P. (1982) Biochim. Biophys. Acta. 681, 474–483.
[16] Rottenberg, H. (1979) Methods Enzymol. 55, 547–569.
[17] Padan, E., Zilberstein, D. and Rottenberg, H. (1976) Eur. J. Biochem. 63, 533–541.
[18] Lolkema, J.S. Hellingwerf, K.J. and Konings, W.N. (1982) Biochim. Biophys. Acta 681, 85–94.
[19] Ghazi, A., Shechter, E., Letellier, L. and Labedan, B. (1981) FEBS Lett. 125, 197–200.
[20] Ghazi, A. and Shechter, E. (1981) Biochim. Biophys. Acta 644, 305–315.
[21] Devor, K.A., Schairer, H.U., Renz, D. and Overath, P. (1974) Eur. J. Biochem. 45, 451–456.
[22] Elferink, M.G.L., Hellingwerf, K.J., Van Belkum, M.J., Poolman, B. and Konings, W.N. (1984) FEMS Microbiol. Lett. 21, 293–298.
[23] Zilberstein, D., Schuldiner, S. and Padan, E. (1979) Biochemistry 18, 669–673.
[24] Ahmed, S. and Booth, I.R. (1981) Biochem. J. 200, 583–589.
[25] Slater, E.C., Berden, J.A. and Herweijer, M.A. (1985) Biochim. Biophys. Acta 811, 217–231.