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Cation/proton antiport systems in *Escherichia coli* K-12, L-form NC7

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Three distinct cation/proton antiport systems have been characterized in L-form NC7 of *Escherichia coli* K-12 by the effects of cations on the pH gradient established by oxidation of lactate or hydrolysis of Mg-ATP in everted membrane vesicles: potassium/proton, sodium/proton and calcium/proton antiport systems. External pH value had significant effects on the Ca^{2+}/proton antiport process and the optimum pH laid in range of 7.5 to 8.0. The dissipation of ΔpH, at pH 7.2, was dependent on the concentration of the externally added Ca^{2+}, and the saturation level was obtained at high concentration (about 10 mM) of Ca^{2+}, whereas at pH 8.0, it was independent on concentration over the range of 1 to 10 mM, and the saturation level was obtained at low Ca^{2+} concentration of about 1 mM.

Introduction

A number of bacteria possess rigid cell walls that gives osmotic protection against swelling. One of entric bacteria, *Escherichia coli* maintains a greater internal osmolarity than the external medium resulting in turgor pressure, which is necessary for cell growth (Epstein and Laimins, 1980). However, when cells lacking rigid cell walls are incubated in an hypotonic solution, they swell and eventually lyse. As organisms without cell walls, Mollicutes (including the families Mycoplasmataceae and Acholeplasmataceae) and many L-forms derived from various bacterial species are well-known. Bacterial L-forms are osmotically fragile, requiring electrolyte such as NaCl (or KCl) or sucrose as osmotic stabilizer in culture medium to survive (King, 1986), whereas osmotic stress, produced by an increase in the osmolarity of the environment inhibit the growth of bacterial cells (Costilow, 1981; Le Rudulier *et al.*, 1984). In hypertonic salt environment the ionic pumps on L-form cells are generally expected to extrude salts and water to regulate cell volume. *E. coli* has three distinct antiport systems which function in the extrusion of cations from the cytosol, namely Ca^{2+}/proton antiport (CHA) system for divalent cations (Brey *et al.*, 1978; Brey and Rosen, 1979; Tsuchiya and Takeda, 1979; Brey *et al.*, 1980; Rosen, 1987), Na^+/proton antiport (NHA) for Na^+ (Brey *et al.*, 1978; Schuldiner and Fishkes, 1978; Bech and Rosen, 1979), and Na^+/proton antiport (KHA) system for monovalent cations (Brey *et al.*, 1978; Brey *et al.*, 1980). By exchanging cations for proton, everted membrane vesicles of *E. coli* mediated uptake of cations,

when an energy donor such as a respiratory substrate or ATP was supplied. This paper will consider the systems responsible for export of ions from *E. coli* K-12, L-form NC7.

Materials and Methods

Preparation of everted membrane vesicles

Escherichia coli K-12, L-form NC7 was grown to stationary phase in a KPY medium (10) supplemented with 0.2% glucose as carbon source. Cultures were harvested, washed twice and suspended in a buffer containing of 10 mM-Tris HCl, pH 7.2, containing 140 mM choline chloride, 5 mM $MgSO_4$, 0.5 mM dithiothreitol and 10% (v/v) glycerol. In other experiments, 0.5 M choline chloride were used, instead of 10% (v/v) glycerol. Everted membrane vesicles were prepared by lysis of cells in a French press. The suspension was centrifuged for 5 min at 10,000 rpm to remove unbroken cells. The supernatant solutions were centrifuged at 100,000 rpm for 30 min. The pelleted membrane vesicles were suspended in the same buffer.

Fluorescence assay

Changes in transmembrane proton gradient (ΔpH) were estimated from the energy-linked quenching of quinacrine fluorescence. Assay were performed in a buffer consisting of 10 mM Tris-HCl, pH 7.2, and 8.0, respectively, containing 1 μM quinacrine, 140 mM choline cloride, 5 mM $MgCl_2$, 0.5 mM dithiothreitol and 100 to 400 μg/ml of membrane protein in a final volume of 2 ml. The salts used were 140 mM choline cloride, 140 mM KCl or 500 mM choline chloride, as noted. Quenching was initiated by addition of Tris-lactate to 10 mM or Mg-ATP to 1 mM. Upon addition of 0.25 μM Carbonylcyanide-*m*-chloro-phenylhydrazone (CCCP) to the above reaction mixture, the fluorescence intensity rapidly recovered due to the dissipation of ΔpH. Nigericin (0.25 μM) instead of CCCP also were used in this experiment. Thus, the intensity recovered and the magnitude of dissipation of ΔpH on addition of cations were expressed as F and ΔF, respectively. Fluorescence was measured by using an Hitachi model 850 fluorescence spectrophotometer with excitation at 420 nm and emmission at 500 nm.

Protein determinations.

Protein was determined by using Bio-Red Protein Assay. Bovine albumin was used as a standard.

Chemicals

The sources of materials used in this work were as follows: Mg-ATP, nigericin and CCCP from Sigma., DL-lactate, dicyclohexylcarbodiimide (DCCD) and quinacrine from Nacalai tesqu. All of the other chemicals were reagent grade and obtained from commercial sources.

Results

Cation/proton antiport system in L-form NC7

In order to determine directly the levels of cation/proton antiporter activities in L-form NC7, we prepared everted membrane vesicles and assayed antiport activities for cations. The establishment of ΔpH was estimated by measuring the fluorescence quenching of quinacrine.

In a Tris-choline buffer, addition of lactate into the membrane vesicles resulted in the fluorescence quenching of quinacrine, reflecting the formation of a pH gradient (acid, interior) established across the membrane of everted vesicles (Fig. 1). Addition of substrates of cation/proton antiporters produces a reversal of quenching by exchange of protons for cations, resulting in the formation of a new steady state ΔpH. Three groups of cations could cause partial dissipation of ΔpH.

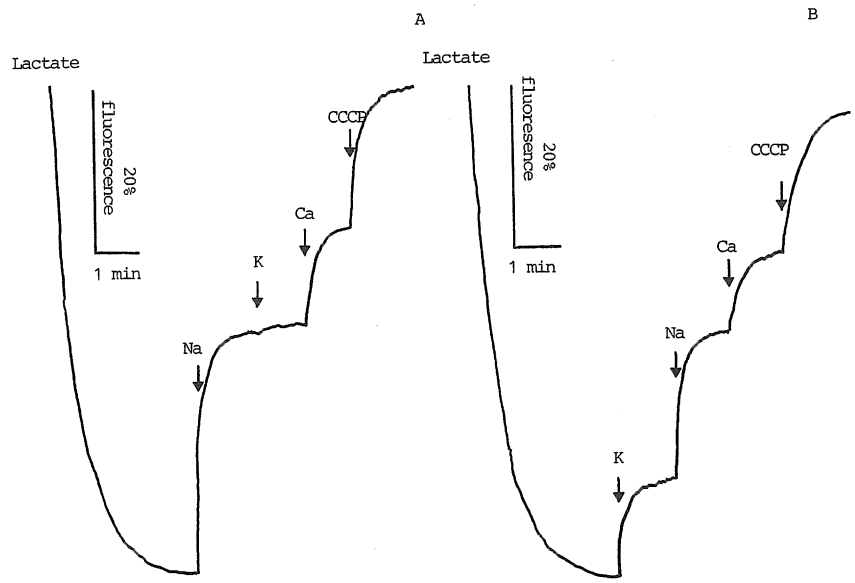

Fig. 1. Antiporter activities of L-form NC7. Activities of cations/proton antiporters were measured by the fluorescence quenching technique. Assay medium contained: 10 mM Tris-HCl (pH 7.2), 0.5 M choline chloride, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 1 μg quinacrine, and 250 μg of protein of membrane vesicles in ml. Quenching was initiated by the addition of Tris/DL-lactate to 10 mM, final concentration. A and B: the cations were added sequentially following formation of ΔpH. At the time indicated by arrow, either KCl, NaCl or $CaCl_2$ was added to yield a final concentration of 10 mM for K^+ and Na^+ or 1 mM for Ca^{2+}.

The specificity of the antiport systems

As shown in Fig. 2a, RbCl was confirmed to dissipate ΔpH in the same way as K^+.

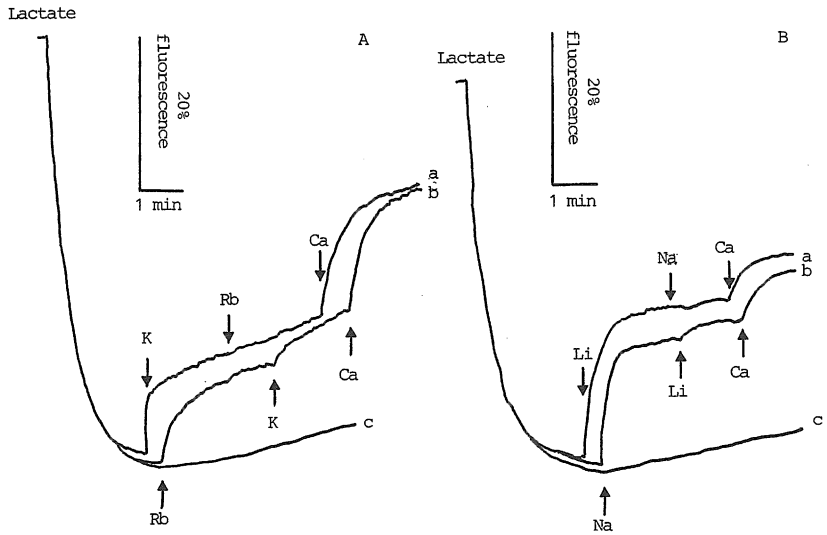

Fig. 2. Specificities of the K^+/H^+ and the Na^+/H^+ antiporters. See Fig. 1 for details. Assay mixtures contained: A, 10 mM Tris-HCl (pH 7.2), 0.5 M choline chloride, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, and 250 μg/ml of membrane protein: B, the same medium as for A but with 0.5 M potassium chloride, instead of 0.5 M choline chloride. a and b: RbCl and LiCl were added at final concentration of 1 mM, and concentrations of other ions were as given in Fig. 1; c: control.

If KCl were added before RbCl, no further effect of Rb^+ on ΔpH was observed. The other cations such as sodium and calcium were able to dissipate ΔpH even in the presence of K^+ and/or Rb^+ (Fig. 2). These results indicate that K^+, Na^+ and Rb^+ are substrates of the K^+/proton antiport (KHA) system. In addition, when Li^+ was used as substrate of the antiport systems, Li^+ could dissipate ΔpH, and subsequent addition of Na^+ had no further effect on ΔpH (Fig. 2b). By contrary, Na^+ inhibited the exchange of Li^+ for proton. These results demonstrate that Na^+ and Li^+ are substrates of the Na^+/proton antiport (NHA) system. Further, Fig. 3 shows that Mn^{2+} shares a common antiporter with Ca^{2+}. Thus, the Ca^{2+}/proton antiporter (CHA) exchanges protons for Ca^{2+} and Mn^{2+}. Quenching of quinacrine fluorescence also was observed by addition of Mg-ATP. When the membrane vesicles mixed with quinacrine were energized with Mg-ATP in the presence of 1 mM, the fluorescence of the dye rapidly quenched. Then, addition of DCCD, an inhibitor of the proton-translocating ATPase, caused a rapid enhancement of fluorescence, as a result of efflux of protons (data not shown).

Effects of pH on the transport systems

By exchanging cations for proton, everted membrane vesicles of L-form NC7 mediated uptake of cations. Of cations tested, the characteristics of calcium transport

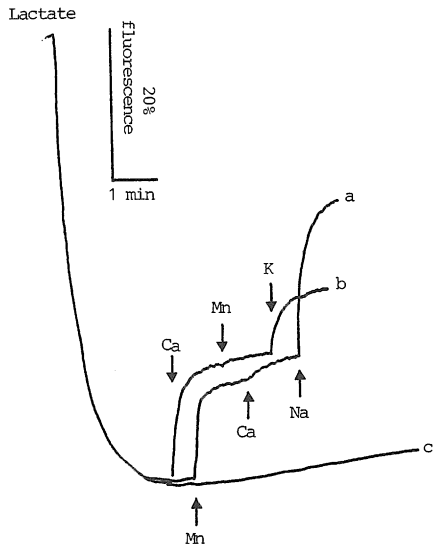

Fig. 3. Specificity of the Ca^{2+}/proton antiporter. See Fig. 1 for details. $CaCl_2$ and $MnCl_2$ were added at final concentration of 5 mM. Concentrations of other ions were as given in Fig. 1. a and b: the cations were added sequentially following formation of ΔpH; c: control.

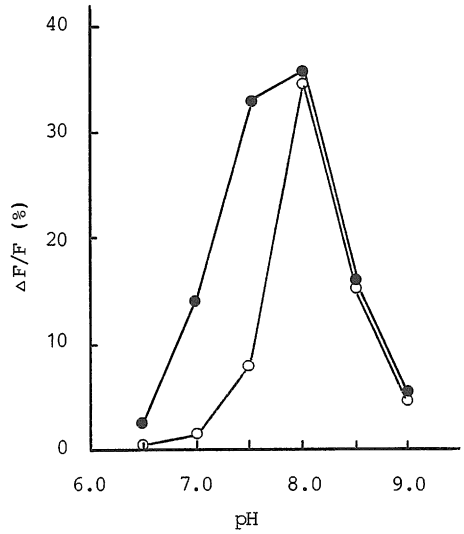

Fig. 4. Effects of external pH on the dissipation of ΔpH of the membrane vesicles in the presence of 1 and 10 mM $CaCl_2$. Assay medium contained: 10 mM MOPS-Tris (pH 6.5 and 7.5) or 10 mM Tris-HCl (pH 7.5 to 9.0), 140 mM choline chloride, 5 mM $MgCl_2$, and 50 μg of membrane protein in ml. Quenching was initiated by the addition of Mg-ATP to 1 mM, final concentration. ○, 1 mM $CaCl_2$: ●, 10 mM $CaCl_2$.

were especially noticed. The rate of ΔF (dissipation of ΔpH) was estimated from the rate at which quinacrine fluorescence attains a new steady state after addition of Ca^{2+}. Fig. 4 shows the magnitude of ΔF/F of the membrane vesicles estimated at various pH values in the presence of $CaCl_2$. Upon increasing the external pH the ΔF/F value increased gradually up to pH 8.0, and there is significant difference between 1 and 10 mM of $CaCl_2$ on dissipation of ΔpH, but in the external pH range above 8.0, the ΔF/F value rapidly decreased and the ratios (ΔF/F) were almost the same in both the cases.

In addition, the initial rates of fluorescence enhancement of quinacrine at pH 7.2 and 8.0, respectively, were measured as a function of calcium concentration (Fig. 5). At assay buffer pH 7.2, the dissipation of ΔpH was dependent on the concentration of externally added Ca^{2+}, and the saturation level was obtained at high $CaCl_2$ concentration of about 10 to 20 mM. On the other hand, at assay buffer of pH 8.0, it was independent of concentration over the range of 1 to 10 mM, and the saturation level was obtained at low Ca^{2+} concentration of about 1 mM.

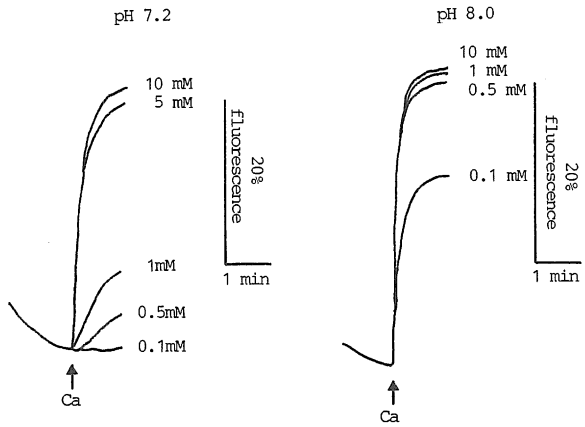

Fig. 5. Concentration dependence of Ca^{2+} dissipation of ΔpH. Assay medium contained: 10 mM Tris-HCl (pH 7.2 and 8.0), 140 mM choline chloride, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, and 50 μg of membrane protein in ml. Quenching was initiated by the addition of Mg-ATP to 1 mM, final concentration, following which $CaCl_2$ were added at the concentrations indicated in the figure and each curve is the result of a separate assay.

Discussion

By osmotic stress such as hypotonic salt solution, active transport of carbohydrate by *E. coli* is drastically inhibited (Roth *et al.*, 1985). They hypothesize that deformation of the membrane by osmotic stress results in conversion of a membrane component of the transport system to a less functional conformation. In L-form, acquired a potency to grow in hypertonic salt medium, it is expected that osmotic stress may result in functionally altered carrier protein. We have attempted here to define cations/proton antiport systems which function in everted vesicles of *E. coli*, L-form NC7. There are at least three antiport systems in everted membrane vesicles prepared from the L-form: The KHA system for K^+, Rb^+ and Na^+, the NHA system for Na^+ and Li^+, and the CHA system for Ca^{2+} and Mn^{2+}. At present, it is still uncertain which antiporter is involved in the regulation of the cytosolic pH and how the intracellular pH is regulated in *E. coli*. Brey *et al.*, (1980) reported that a more likely function of the KHA system would be as a regulator of intracellular pH. In *E. coli*, the involvement of the NHA system in pH regulation has been suggested by several workers (Padan *et al.*, 1981; Booth, 1985; McMorrow *et al.*, 1989) and there have reported on mutant of *E. coli* with defective Na^+/proton antiporter that does not grow at alkaline pH (Ishikawa *et al.*, 1987). The other antiporter system found in many bacteria is the Ca^{2+}/proton antiporter. However, the molecular mechanism of active transport of Ca^{2+} in *E. coli* membrane vesicle is yet unclear. Recently, we reported that *E. coli* K-12 L-form NC7 grows in KPY or NaPY medium and requires for optimal growth external Ca^{2+} or 0.1 mM (in KPY medium) or 1.0 mM (in MaPY medium) (Onoda *et al.*, 1987; Onoda and

Oshima, 1988). When the initial rates of fluorescence enhancement of quinacrine at pH 7.2 and 8.0, respectively, were measured as a function of calcium concentration, at pH 7.2, the dissipation of ΔpH was dependent on the concentration of the externally added Ca^{2+}, while at pH 8.0, the action of calcium is independent of concentration over the range of 1 to 10 mM. These results suggested that the affinity of Ca^{2+} to carrier protein(s) and its activity on membrane vesicles in hypertonic salt solution may be regulated by external pH. Since membrane vesicles are easily obtained from bacterial L-forms lacking rigid cell walls, they would be useful tools for studying the mechanisms of ions transport systems.

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