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Inhibition of Melibiose Transporter by Amiloride in *Escherichia coli*

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Amiloride inhibited the active transport of melibiose via the melibiose transporter in *Escherichia coli*. Since amiloride is known to inhibit the Na^+/H^+ antiporter which is essential for the establishment of an electrochemical potential of Na^+ that is the driving force for melibiose transport, we tested the effect of amiloride on the activity of the melibiose transporter itself. Amiloride inhibited the melibiose counterflow. Also, inhibition of Na^+ uptake induced by melibiose influx and some inhibition of H^+ uptake induced by melibiose influx were observed. These results indicate that amiloride directly inhibits the melibiose transporter, perhaps by competing with Na^+. It seems that the Na^+ binding site and the H^+ binding site in the melibiose transporter are somehow different from each other judging from the difference in the inhibition pattern of amiloride.

The melibiose transporter of *Escherichia coli* mediates cation/galactoside symport (1). This transporter has two remarkable features. One is the variety of cations that can be used for the symport. The coupling cation can be either Na^+, H^+, or Li^+. The other is the versatility of the coupling cation. Coupling cations vary depending on the substrate transported (1). Na^+ is the most effective coupling cation for melibiose transport, followed by H^+ and Li^+ (Li^+ is a poor coupling cation). Either Na^+ or Li^+, but not H^+, is utilized for methyl-β-thiogalactoside (TMG) transport. Na^+, H^+ or Li^+ can be used for methyl-α-galactoside transport. Thus, this transport system is well suited for the analysis of the mechanism of cation coupling. Mutants showing altered cation specificity were isolated and characterized (2, 3, 4). These mutants provided valuable insights into the cation coupling mechanism.

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In addition to these biochemical analyses of the melibiose transporter, a genetic analysis has been made. The *melB* gene encoding the melibiose transporter was cloned (5) and sequenced (6). Thus, it became possible to elucidate role(s) of amino acid residues in the melibiose transporter.

Recently, the melibiose transporter protein has been purified and further characterized (7).

If there is a specific inhibitor of the symporter, it is useful for investigating the mechanism of the symport and structure-function relationship in the symporter. The diuretic drug amiloride is known as a specific inhibitor of the Na^+-related transporter in several cell types. It inhibits the Na^+ channel (8), the Na^+/H^+ antiporter of *E. coli* (9) and of *Vibrio parahaemolyticus* (10), the Na^+/H^+ exchanger (NHE) of mammalian cells (11) and the Na^+-driven flagellar motor in bacteria (12). Thus, amiloride may also inhibit the melibiose transporter. Here we report the effect of amiloride on the melibiose transporter.

MATERIALS AND METHODS

Bacterium and growth. *E. coli* DW1/pKKMB (13) which lacks both the lactose transporter gene and the α-galactosidase gene was used. Cells were grown at 37°C in a minimal medium (14) (Na^+ salts were replaced with K^+ salts) supplemented with 1% tryptone and 10 mM melibiose. Cells were harvested at the late logarithmic phase of growth.

Transport assays. Active transport of [^{3}H]melibiose was measured as described previously (15). When necessary, amiloride was added to the assay mixture. Melibiose counterflow was measured as follows. After harvesting, cells were washed with the minimal medium, and suspended in the medium containing 5 mM 2,4-dinitrophenol and 20 mM melibiose. The cell suspension was shaken for 7 hr at 37°C to starve energy and to load the cells with melibiose. The cells were then harvested, washed twice with the minimal medium and resuspended in 0.1 M MOPS (3-morpholinopropanesulfonic acid)-TMAH (tetramethylammonium hydroxide), pH 7.0, and 10 mM NaCl containing either no amiloride or 3 mM amiloride at 4°C. The counterflow assay was initiated by diluting (50-fold) the cell suspension into the assay mixture consisting of 0.1 M MOPS-TMAH, pH 7.0, 10 mM NaCl, 50 μM CCCP (carbonylcyanide-*m*-chlorophenylhy-

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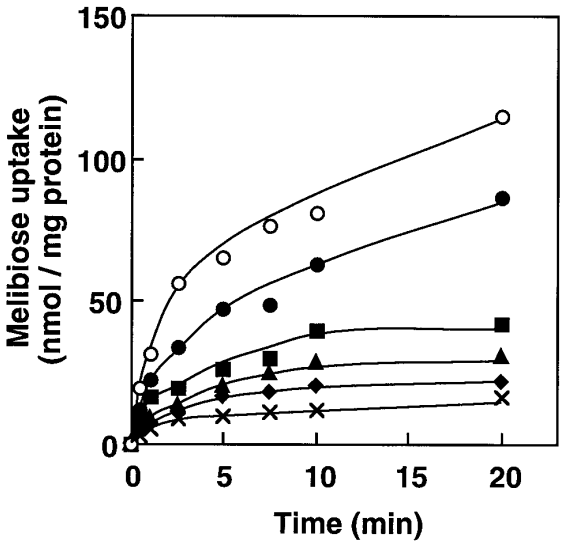

FIG. 1. Inhibition of melibiose active transport by amiloride in *E. coli* cells. Transport was measured in an assay mixture containing 0.1 M MOPS-TMAH, pH 7.0, 10 mM NaCl, 2 mM $MgSO_4$, 10 mM potassium lactate, and the indicated concentrations of amiloride at 25°C. The transport assay was initiated upon addition of [^{3}H]-melibiose (final concentration 50 μM). Symbols: ○, control; ●, 0.2 mM amiloride; ■, 0.5 mM amiloride; ▲, 1 mM amiloride; ◆, 2 mM amiloride; ×, 3 mM amiloride.

drazone) and 50 μM [^{3}H]melibiose containing either no amiloride or 3 mM amiloride.

Measurement of Na^+ uptake and H^+ uptake. Uptake of Na^+ or H^+ induced by melibiose influx was measured as described previously (1).

Protein assay. Protein contents were determined by Lowry's method with bovine serum albumin as a standard (16).

Materials. Amiloride was obtained from Sigma Co. Radioactive [^{3}H]melibiose was from ROTEM Co. (Israel). All other reagents were obtained from commercial sources.

RESULTS AND DISCUSSION

E. coli cells possess two distinct pathways for melibiose transport, the lactose transporter and the melibiose transporter (15). To test the effect of amiloride on the melibiose transporter, we used *E. coli* DW1/pKKMB which lacks the lactose transporter. This mutant also lacks α-galactosidase. Therefore, melibiose is not metabolized in cells of this mutant. First we tested the effect of various concentrations of amiloride on the time course of melibiose active transport (Na^+/melibiose symport). Amiloride inhibited the active transport at 0.2 to 3 mM (Fig. 1).

An electrochemical potential of Na^+ across the cytoplasmic membrane is necessary to drive active transport of melibiose via the melibiose transporter. Two systems are involved in the formation of the electrochemical potential of Na^+ in the *E. coli* cell membrane, the respiratory chain and the Na^+/H^+ antiporter. The respiratory chain establishes an electrochemical potential of H^+ across the membrane. Thereafter, the electrochemical potential of H^+ is converted to that of Na^+ by the function of Na^+/H^+ antiporter. Although amiloride did not significantly affect the respiratory chain (data not shown), it has been reported that the Na^+/H^+ antiporter of *E. coli* was inhibited by amiloride (9). We confirmed such inhibition (data not shown). Thus, it is very likely that amiloride reduces the magnitude of the electrochemical potential of Na^+. It is therefore necessary to clarify whether amiloride directly inhibits the melibiose transporter.

The melibiose counterflow assay is an appropriate method for measuring the activity of the melibiose transporter without the involvement of the Na^+/H^+ antiporter. We reported previously that the melibiose counterflow via the melibiose transporter was dependent on Na^+ (17). In the present study, we tested the effect of amiloride on the melibiose counterflow in energy-starved cells in the presence of 10 mM NaCl. We added CCCP to the assay mixture to reduce the residual active transport due to residual energy sources. As shown in Fig. 2, we observed melibiose counterflow activity in the control cells (no amiloride). The activity was completely inhibited by 3 mM amiloride. Thus, it seems that amiloride inhibits the melibiose transporter directly.

To confirm the direct effect (inhibition) of amiloride on the melibiose transporter, we tested the effect of amiloride on the Na^+ influx into cells elicited by passive melibiose influx (Na^+/melibiose symport). Amiloride inhibited the Na^+ influx at 1 to 3 mM (Fig. 3). Considerable inhibition by amiloride was observed at 0.2 to 0.5

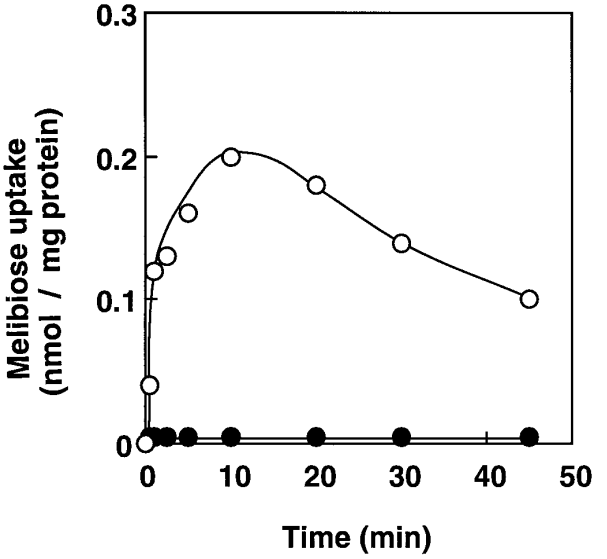

FIG. 2. Inhibition of melibiose counterflow by amiloride. Melibiose counterflow was measured in an assay mixture containing 0.1 M MOPS-TMAH, pH 7.0, 10 mM NaCl, 50 μM CCCP, and 50 μM [^{3}H]melibiose either in the absence (○) or in the presence of 3 mM amiloride (●) at 25°C.

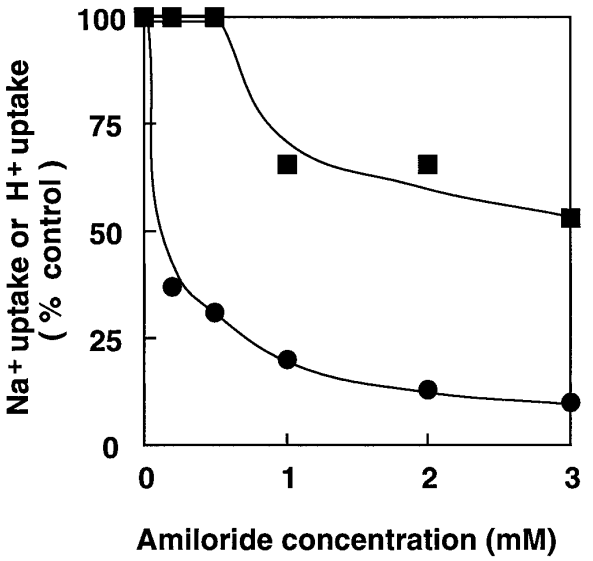

FIG. 3. Effects of amiloride on Na^+ uptake or H^+ uptake induced by melibiose influx. Na^+ uptake was measured in an assay mixture containing 0.1 M Tricine-TMAH, pH 8.0, 25 μM NaCl, and the indicated concentrations of amiloride at 25°C. Melibiose (final concentration 5 mM) was added to the assay mixture to induce Na^+ uptake under anaerobic conditions. The initial velocity of the Na^+ uptake was calculated (●). The control value for the Na^+ uptake (without amiloride) was 8.8 ng-ions Na^+/min per mg cell protein. H^+ uptake was measured in an assay mixture containing 150 mM KCl, 2 mM $MgSO_4$, and the indicated concentrations of amiloride at 25°C. Melibiose (final concentration 5 mM) was added to the assay mixture to induce H^+ uptake under anaerobic conditions. Initial velocity of the H^+ uptake was calculated (■). Control value for H^+ uptake (without amiloride) was 16 ng-ions H^+/min per mg cell protein.

mM. Thus, we conclude that amiloride is a potent inhibitor of Na^+/melibiose symport via the melibiose transporter. We also tested the effect of amiloride on H^+/melibiose symport. We observed some inhibition of H^+ influx elicited by melibiose influx by amiloride at 1 to 3 mM (Fig. 3). No inhibition was detected at 0.2 to 0.5 mM. These results suggest that Na^+ and H^+ do not share the same binding site in the melibiose transporter. This conclusion is in agreement with previous observations that a mutant-type melibiose transporter which lacked the ability to couple with H^+ showed normal Na^+-coupling (2, 3). Perhaps amiloride competes with Na^+ for the Na^+ binding site, as shown in the flagellar motor (12). It seems that the binding of amiloride to the Na^+ site or its vicinity does not significantly affect the H^+/melibiose symport activity.

A domain for amiloride binding has been suggested in the Na^+/H^+ exchanger (NHE) (11). We found that one region in the melibiose transporter possesses a sequence very similar to the suggested amiloride binding domain of the NHE (data not shown). A site-directed mutagenesis study in this region of the melibiose transporter may by useful for the analysis of the action of amiloride on the melibiose transporter and for analysis of the structure-function relationship in this transporter.

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REFERENCES

1. Tsuchiya, T., and Wilson, T. H. (1978) *Membr. Biochem.* **2,** 63–79.
2. Niiya, S., Yamasaki, K., Wilson, T. H., and Tsuchiya, T. (1982) *J. Biol. Chem.* **257,** 8902–8906.
3. Yazyu, H., Shiota, S., Futai, M., and Tsuchiya, T. (1985) *J. Bacteriol.* **162,** 933–937.
4. Kawakami, T., Akizawa, Y., Ishikawa, T., Shimamoto, T., Tsuda, M., and Tsuchiya, T. (1988) *J. Biol. Chem.* **263,** 14276–14280.
5. Hanatani, M., Yazyu, H., S-Niiya, S., Moriyama, Y., Kanazawa, H., Futai, M., and Tsuchiya, T. (1984) *J. Biol. Chem.* **259,** 1807–1812.
6. Yazyu, H., S-Niiya, S., Shimamoto, T., Kanazawa, H., Futai, M., and Tsuchiya, T. (1984) *J. Biol. Chem.* **259,** 4320–4326.
7. Pourcher, T., Leclercq, S., Brandolin, G., and Leblanc, G. (1995) *Biochemistry* **34,** 4412–4420.
8. Kleyman, T. R., and Cragoe, E. J., Jr. (1988) *J. Membr. Biol.* **105,** 1–21.
9. Mochizuki-Oda, N., and Oosawa, F. (1985) *J. Bacteriol.* **163,** 395–397.
10. Kuroda, T., Shimamoto, T., Inaba, K., Tsuda, M., and Tsuchiya, T. (1994) *J. Biochem.* **115,** 1162–1165.
11. Counillon, L., Franchi, A., and Pouyssegur, J. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90,** 4508–4512.
12. Atsumi, T., Sugiyama, S., Cragoe, E. J., and Imae, Y. (1990) *J. Bacteriol.* **172,** 1634–1639.
13. Botfield, M. C., and Wilson. T. H. (1989) *J. Biol. Chem.* **264,** 11643–11648.
14. Tanaka, S., Lerner, S. A., and Lin, E. C. C. (1967) *J. Bacteriol.* **93,** 642–648.
15. Lopilato, J., Tsuchiya, T., and Wilson, T. H. (1978) *J. Bacteriol.* **134,** 147–156.
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
17. Tsuchiya, T., Ottina, K., Moriyama, Y., Newman, M. J., and Wilson. T. H. (1982) *J. Biol. Chem.* **257,** 5125–5128.