Correlation between the respiration-driven Na⁺ pump and Na⁺-dependent amino acid transport in moderately halophilic bacteria

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The effect of Na⁺ on amino acid uptake by moderately halophilic bacteria was examined using α -aminoisobutyric acid (AIB) as a nonmetabolizable amino acid analogue. Of the eight moderate halophiles investigated, the six Gram-negative bacteria that have a respiration-driven Na⁺ pump specifically required Na⁺ for AIB uptake. On the other hand, the two Gram-positive bacteria that have no respiration-driven Na⁺ pump showed no requirement for Na⁺ for AIB uptake. Thus, the mode of energy coupling to amino acid transport was quite different between the Gram-negative and Gram-positive moderate halophiles tested: the former, but not the latter, utilized Na⁺ circulation for the active uptake of AIB.

Introduction

Halophilic bacteria are divided into three categories based on their salt requirement for optimal growth (Kushner, 1978). Slightly halophilic marine bacteria grow best in media containing 0.2–0.5 M-NaCl, moderately halophilic bacteria have a growth optimum at 0.5–2.5 M-NaCl, and extremely halophilic bacteria at 2.5–5.2 M-NaCl. Marine bacteria (Niven & MacLeod, 1980; Tokuda *et al.*, 1982) and extreme halophiles (MacDonald *et al.*, 1977; Lanyi, 1979) are known to require a sodium-motive force ($\Delta\mu_{\text{Na}^+}$) for the active uptake of amino acids. The moderate halophile *Vibrio costicola* requires a $\Delta\mu_{\text{Na}^+}$ for active uptake of α -aminoisobutyric acid (AIB) (Hamaide *et al.*, 1984). Therefore, halophilic bacteria that require Na⁺ for optimal growth are generally considered to utilize $\Delta\mu_{\text{Na}^+}$ as the driving force of amino acid uptake. Recently, we have demonstrated that among moderately halophilic bacteria from diverse origins, Gram-negative, but not Gram-positive, halophiles have a respiration-driven Na⁺ pump (Unemoto *et al.*, 1992). At present, amino acid transport has been investigated in only a few moderate halophiles (Hamaide *et al.*, 1984). Since the mode of energy generation in moderate halophiles is quite

different between Gram-negative and Gram-positive bacteria, we were interested in energy coupling to amino acid transport in these bacteria. Therefore, using AIB as a nonmetabolizable amino acid analogue, the effect of Na⁺ on the active uptake of AIB by these moderate halophiles was studied with special reference to the presence or absence of a respiration-driven Na⁺ pump.

Methods

Bacterial species and culture conditions. The moderately halophilic bacteria used in this study and their culture conditions were described previously (Unemoto *et al.*, 1992). Each bacterium was grown in the presence of 1.0 M-NaCl and harvested at the late exponential phase of growth by centrifugation. The cells were washed twice with a medium containing 25 mM-HEPES/Tris (pH 7.5), 1.0 M-KCl and 10 mM-MgSO₄, and suspended in this medium at a concentration of 5–10 mg cell protein ml⁻¹. The cell suspension was stored on ice until use. Protein was determined by the Lowry method with bovine serum albumin as a standard.

Amino acid uptake. AIB was used as a nonmetabolizable amino acid analogue. To 100 μ l of the reaction mixture containing 25 mM-buffer, 0.1 mM-[³H]AIB [20 μ Ci μ mol⁻¹ (740 kBq μ mol⁻¹); New England Nuclear] and 0.2–2.0 M-NaCl or KCl, 2 μ l of the cell suspension was added at 37 °C. At time intervals, the reaction was terminated by the addition of 1.0 ml 1.0 M-NaCl and by immediate filtration on a MF Millipore filter (pore size 0.45 μ m). The filter was washed once with 1.0 ml 1.0 M-NaCl within 10 s. The radioactivity was determined with 3 ml of Bray's scintillation liquid. The radioactivity retained on the filter in the absence of cells served as the blank. To prevent the lysis of cells at low salt concentrations, 10 mM-MgSO₄ was included in the reaction mixture. The following buffers were used at different pH values: 25 mM-MES/Tris (pH 6.0–6.5), 25 mM-HEPES/Tris (pH 7.0–7.5), 25 mM-Tricine/Tris (pH 8.0–8.5) and 25 mM-Tris/CHES (pH 9.0).

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Abbreviations: AIB, α -aminoisobutyric acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazide; $\Delta\mu_{\text{H}^+}$, electrochemical potential gradient of H⁺; $\Delta\mu_{\text{Na}^+}$, electrochemical potential gradient of Na⁺.

Fig. 1

Fig. 1. Effects of NaCl and KCl on the active uptake of AIB by *H. variabilis*. The initial rate of AIB uptake was assayed at pH 7.5 in the presence of NaCl (●) or KCl (○).

Fig. 2

Fig. 2. Effects of CCCP and pH on the AIB uptake of *H. variabilis*. (a) Initial rate of AIB uptake assayed in the presence of 1.0 M-NaCl and various concentrations of CCCP at pH 6.5 (○) and 8.5 (●). (b) AIB uptake assayed in the presence of 1.0 M-NaCl at various pH values. ○, No addition; ●, 10 μM-CCCP.

Results

Effect of NaCl on the active uptake of AIB

Optimal conditions for the assay of AIB uptake were examined using *Halovibrio variabilis* (DSM 3051^T). In the presence of 1.0 M-NaCl, AIB uptake increased linearly with incubation time at least for 5 min. Therefore the reaction was terminated at 3 min to measure the initial velocity of AIB uptake. As shown in Fig. 1, *H. variabilis* required Na⁺ for the active uptake of AIB with an optimal concentration of 0.6–1.2 M-NaCl. The rate of AIB uptake decreased at higher concentrations. No significant AIB uptake was observed with KCl as a replacement for NaCl at any concentrations. In the steady state, AIB was accumulated to about 100 nmol (mg protein)⁻¹. Assuming that the internal cell volume is similar to that of *V. costicola* [3 μl (mg protein)⁻¹] (Udagawa *et al.*, 1986), the intracellular concentration of AIB was calculated to be 33 mM, which was 330-fold higher than that of the external medium. When 100 mM unlabelled AIB was added in the steady state, the radioactive AIB accumulated within the cells was chased from the cells (data not shown). Apparently, *H. variabilis* required Na⁺ for the active uptake of AIB.

Table 1 shows the effects of 1.0 M-NaCl and KCl on AIB uptake by various moderate halophiles at pH 6.5 and 8.5. Of the eight organisms, the six Gram-negative bacteria *H. variabilis*, *Deleya halophila* (CCM 3662^T), *V. costicola* (NCMB 701^T), *Pseudomonas beijerinckii* (ATCC 19372), *Ps. halosaccharolytica* (ATCC 29423) and an unidentified halophile (NRCC 41227) required Na⁺ for the active uptake of AIB at acidic and alkaline pH values. On the other hand, the two Gram-positive bacteria *Marinococcus halophilus* (CCM 2706^T) and *Micrococcus varians* subsp. *halophilus* (ATCC 21971) showed nearly the same activity with either NaCl or KCl, exhibiting no re-

quirement for Na⁺ for the active uptake of AIB. In our previous paper (Unemoto *et al.*, 1992), the above six Gram-negative, but not the two Gram-positive, halophiles were demonstrated to have a respiration-driven Na⁺ pump. Therefore, these results strongly suggest that moderate halophiles that have a respiration-driven Na⁺ pump require Na⁺ for the active uptake of AIB.

Effect of a proton conductor on the active uptake of AIB

In the marine *V. alginolyticus*, the respiration-driven Na⁺ pump extrudes Na⁺ against its electrochemical gradient and generates a proton-conductor-resistant membrane potential especially at alkaline pH (Tokuda & Unemoto, 1982). At acidic pH, the Δμ_{Na⁺} is mainly generated by a Δμ_{H⁺}-driven Na⁺/H⁺ antiporter. If amino acid uptake is driven by Δμ_{Na⁺}, it may be inhibited by a proton conductor at acidic, but not at alkaline, pH. Fig. 2(a) shows the effect of a proton conductor, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), on AIB uptake by *H. variabilis* at pH 6.5 and 8.5 in the presence of 1.0 M-NaCl. AIB uptake was almost completely inhibited by 5 μM-CCCP at pH 6.5, whereas at pH 8.5 more than 20 μM-CCCP was required to inhibit AIB uptake by 50%. Fig. 2(b) shows the effects of pH and 10 μM-CCCP on AIB uptake. In the absence of CCCP, the rate of AIB uptake was almost unaffected by medium pH. However, the AIB uptake was strongly inhibited by 10 μM-CCCP at acidic, but not at alkaline, pH values. These results were essentially the same as those obtained with the marine *V. alginolyticus* (Tokuda & Unemoto, 1981). Since *H. variabilis* has the same type of respiration-driven Na⁺ pump as *V. alginolyticus* (Unemoto *et al.*, 1992), the CCCP-resistant AIB uptake at alkaline pH was probably due to the direct generation of Δμ_{Na⁺} by the

Table 1. Effects of 1.0 M-NaCl and KCl on the AIB uptake of moderate halophiles at pH 6.5 and 8.5

The initial velocity was determined from the linear part of AIB uptake and results presented are mean values of three to four separate experiments.

Moderate halophile	AIB uptake [nmol min ⁻¹ (mg protein) ⁻¹]			
	pH 6.5		pH 8.5	
	NaCl	KCl	NaCl	KCl
<i>Halovibrio variabilis</i> (DSM 3051 ^T)	14.9	0.89	15.2	0.77
<i>Deleya halophila</i> (CCM 3662 ^T)	10.3	1.09	9.37	0.89
<i>Vibrio costicola</i> (NCMB 701 ^T)	1.34	0.03	1.09	0.02
<i>Pseudomonas beijerinckii</i> (ATCC 19372)	1.36	0.06	0.74	0.01
<i>Ps. halosaccharolytica</i> (ATCC 29423)	3.03	0.03	2.34	0.02
Unidentified halophile (NRCC 41227)	0.50	0.04	0.42	0.01
<i>Marinococcus halophilus</i> (CCM 2706 ^T)	2.12	2.22	1.85	1.74
<i>Micrococcus varians</i> subsp. <i>halophilus</i> (ATCC 21971)	2.44	2.97	1.46	1.49

respiration-driven Na⁺ pump. With respect to *V. costicola*, detailed studies on the generation of $\Delta\mu_{\text{Na}^+}$ have already been reported (Udagawa *et al.*, 1986). For the other Gram-negative moderate halophiles examined in Table 1, it was also confirmed that AIB uptake was resistant to 10 μM -CCCP at alkaline, but not acidic, pH (data not shown). These results strongly suggest that moderate halophiles that have a respiration-driven Na⁺ pump utilized $\Delta\mu_{\text{Na}^+}$ for the active uptake of AIB. In this connection, it is interesting to note that the pH-dependent resistance curve of AIB uptake against CCCP is very similar to the pH-dependent resistance curve of growth rate of *V. alginolyticus* against CCCP (see Fig. 3 in Tokuda & Unemoto, 1983).

Discussion

The Na⁺-dependent active transport of various nutrients has been reported in the marine *Alteromonas haloplanktis* and *V. fischeri* (Droniuk *et al.*, 1987), and *Deleya aesta* (Berthelet & MacLeod, 1991). The marine *V. alginolyticus* also requires Na⁺ for the active uptake of 20 amino acids including AIB (Unemoto *et al.*, 1990). Using AIB as the substrate, $\Delta\mu_{\text{Na}^+}$ was shown to act as the direct driving force in *A. haloplanktis* (Niven & MacLeod, 1980) and in *V. alginolyticus* (Tokuda *et al.*, 1982). The moderately halophilic *V. costicola* was also shown to require $\Delta\mu_{\text{Na}^+}$ for AIB uptake (Hamaide *et al.*, 1984). Thus, the Na⁺-coupled transport systems were generally considered to be common in the marine and moderately halophilic bacteria. However, in this paper it was found

that all moderate halophiles do not necessarily require Na⁺ for the active uptake of AIB. Indeed, the two Gram-positive moderate halophiles that have no respiration-driven Na⁺ pump showed no requirement for Na⁺ for the active uptake of AIB. Thus, these moderate halophiles are unlikely to utilize $\Delta\mu_{\text{Na}^+}$ for the active uptake of AIB.

Except for the concentration of NaCl required for optimal growth (1.0–2.0 M), the six Gram-negative moderate halophiles examined are very similar to the marine bacteria in the following properties: (1) the NaCl-washed cells quickly lyse in a hypotonic medium; (2) they have Na⁺-dependent NADH-quinone reductase in the respiratory chain that functions as an electrogenic Na⁺ pump; (3) the active uptake of AIB is dependent on Na⁺ and thus driven by $\Delta\mu_{\text{Na}^+}$. These three properties are characteristic of marine bacteria and discriminate between marine and terrestrial bacteria. Thus, the Gram-negative moderate halophiles examined are closely related to the marine bacteria, suggesting that these moderate halophiles originated from marine environments. However, the two Gram-positive moderate halophiles examined are resistant to hypotonic lysis, do not have any respiration-driven Na⁺ pump, and exhibit no requirement for Na⁺ for AIB uptake. Since these Gram-positive halophiles have none of the properties characteristic of marine bacteria, they are unlikely to originate from marine environments. Gram-positive halophiles constitute a minor proportion of the bacteria found in marine and high salt environments and only two species were examined in this study. Recently, Bejar *et al.* (1992) classified 23 strains of moderately halophilic endospore-forming Gram-positive rods into two groups, which were different from those of other previously described *Bacillus* species. Therefore, it is interesting to further investigate other Gram-positive moderate halophiles from the bioenergetic point of view to generalize our results.

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