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Na^+/SOLUTE SYMPORT IN MEMBRANE VESICLES FROM *BACILLUS ALCALOPHILUS*

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The characteristics of α-aminoisobutyric acid translocation were examined in membrane vesicles from obligately alkalophilic *Bacillus alcalophilus* and its non-alkalophilic mutant derivative, KM23. Vesicles from both strains exhibited α-aminoisobutyric acid uptake upon energization with ascorbate and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine. The presence of Na^+ caused a pronounced reduction in the K_m for α-aminoisobutyric acid in wild-type but not KM23 vesicles; the maximum velocity (V) was unaffected in vesicles from both strains. Passive efflux and exchange of α-aminoisobutyric acid from wild-type vesicles were Na^+-dependent and occurred at comparable rates (with efflux slightly faster than exchange). This latter observation suggests that the return of the unloaded carrier to the inner surface is not rate-limiting for efflux. The rates of α-aminoisobutyric acid efflux and exchange were also comparable in KM23 vesicles, but were Na^+-independent. Furthermore, in vesicles from the two strains, both efflux and exchange were inhibited by generation of a transmembrane electrochemical gradient of protons, outside positive. This suggests that the ternary complex between solute, carrier, and coupling ion bears a positive charge in both strains even though the coupling ion is changed. Evidence from experiments with an alkalophilic strain that was deficient in L-methionine transport indicated that the porters, i.e., the solute-translocating elements, used by non-alkalophilic mutants are not genetically distinct from those used by the alkalophilic parent; that is, the change in coupling ion cannot be explained by the expression of a completely new set of Na^+-independent, H^+-coupled porters upon mutation of *B. alcalophilus* to non-alkalophily.

Introduction

Obligately alkalophilic bacteria generally employ an Na^+/solute symport mechanism for ion-coupled solute transport [1–5] rather than the H^+/solute symport more commonly found in bacteria [6–11]. The latter coupling of solute transport to H^+ movement represents energization of transport directly by the electrochemical proton gradient, $\Delta\bar{\mu}_{H^+}$, as proposed by Mitchell [7,12,13]. Mitchell [13] also proposed that a secondary Na^+/H^+ antiporter could convert the $\Delta\bar{\mu}_{H^+}$, all or in part, to an electrochemical gradient of sodium ions. The Na^+ gradient thus obtained might energize some bacterial transport systems. Na^+ is the usual coupling ion for porters in eukaryotic tissues that possess ($Na^+ + K^+$)-ATPase activity [14], but in conventional bacteria, Na^+/solute symport is the interesting exception (see, for example, Refs. 15–19) even in cells in which Na^+/H^+ antiport activity has been documented. Only in selectively pressured species, such as the obligate alkalophiles, *Halobacterium* [20,21], or marine pseudomonads [22], has Na^+ evolved as the dominant coupling ion.

The selective pressure on the akalophiles is the requirement for a cytoplasmic pH that is acidified relative to optimal external pH values of 10.5–11.5. The vital function of pH homeostasis is apparently

Abbreviations: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; $\Delta\bar{\mu}_{H^+}$, electrochemical proton gradient.

served by a Na^+/H^+ antiporter [4,23,24]. In *Bacillus alcalophilus*, this antiporter's activity, together with primary proton extrusion by the respiratory chain, results in chemical gradients of protons, $[H^+]_{in} > [H^+]_{out}$, and of sodium ions, $[Na^+]_{out} > [Na^+]_{in}$, and a transmembrane electrical potential, outside positive [24]. Interestingly, non-alkalophilic mutant strains (e.g., KM23) which have lost Na^+/H^+ antiporter activity also lose Na^+-coupling to solute symport [5,23]. This loss is apparently a primary consequence of the mutation. The initial uptake rates and passive solute efflux rates are both affected; moreover, monensin, an artificial Na^+/H^+ exchanger, fails to restore Na^+-coupled uptake of efflux [5,23]. We have advanced the hypothesis that there is an element involved in Na^+-translocation that is common to the Na^+/H^+ antiporter and Na^+-coupled symporters; non-alkalophic strains could have lost that element, although they still possess active solute porters that function at neutral pH [5,23,25]. Evidence from studies of whole cells indicates that non-alkalophilic strain KM23 can couple solute transport to proton movement [23]. The current study was undertaken to further elucidate and compare the characteristics of cation/solute symport, especially in membrane vesicles, of wild-type *B. alcalophilus* and its nonalkalophilic derivative KM23. We have compared some of the kinetic properties of α-aminoisobutyric acid uptake, passive efflux and exchange. Also among the goals was the demonstration that the porters that function in KM23 are really the same porters that exist in the wild type, albeit altered in coupling ion.

Methods

Bacillus alcalophilus (ATCC 27647) and KM23, its non-alkalophilic derivative, were grown on L-malate-containing media at pH 10.5 and 6.8, respectively [3,23]. Both media contained a final concentration of 50 mM L-malate and 0.1% yeast extract. A mutant of *B. alcalophilus* unable to transport L-methionine was obatined by mutagenesis with ethylmethane sulfonate [26] and plating at pH 10.5 in the presence of 5 mM DL-ethionine, an analogue of L-methionine [27]. Colonies were picked and grown in L-malate broth culture at pH 10.5, and screened for L-methionine uptake. Mid-logarithmic phase cells of the wild type and possible mutants were harvested by centrifugation (20 000 × *g*), washed twice with 25 mM potassium carbonate buffer (pH 9.0) and suspended to approx. 0.05 mg cell protein/ml. Uptake of L-[^{14}C]-methionine was assayed by the filtration method used for α-aminoisobutyric acid uptake [3]. L-Malate (10 mM) was present in all suspensions as the energy source and cells were aerated by vigorous mixing. Half the cell suspensions contained 10 mM KCl while the other half contained 10 mM NaCl. Radioactive L-methionine was added to initiate the reaction and samples were removed at intervals to establish the initial rate of L-methionine uptake. *B. alcalophilus* wild type took up L-methionine at a rate of 0.79 nmol/min per mg cell protein in the absence of Na^+, and 1.67 nmol/min per mg cell protein in the presence of Na^+ at pH 9.0. One of the mutants of *B. alcalophilus* resistant to DL-ethonine, designated MM7, took up L-methionine at a much lower rate: 0.06 nmol/min per mg cell protein in the presence or absence of Na^+ at pH 9.0.

When uptake of L-methionine was assayed at pH 7.0, the cells were washed and suspended in 25 mM potassium phosphate, plus 10 mM potassium L-malate, with the addition of either 10 mM KCl or 10 mM NaCl.

Membrane vesicles were prepared from lysozyme-induced protoplasts of *B. alcalophilus* or KM23 [24,28]. Vesicles of *B. alcalophilus* were routinely prepared and stored in 100 mM potassium carbonate (pH 9.0) plus 10 mM $MgSO_4$, whereas vesicles of KM23 were prepared and stored in 100 mM potassium phosphate buffer (pH 7.0) and 10 mM $MgSO_4$. Uptake of α-aminoisobutyric acid by vesicles was assayed by filtration upon energization with 20 mM ascorbate/2 mM TMPD [5]. After 1 min incubation at 30°C with oxygenation in the presence of the electron donor, uptake was initiated by the addition of 40 μM α-amino[^{14}C]isobutyric acid. The reaction mixture contained 1 mg membrane protein/ml. Samples were removed, filtered, washed and assayed for radioactivity [5].

Passive efflux and exchange of solute was studied in vesicles using techniques developed by Kaback and his associates [29–31]. Efflux from *B. alcalophilus* vesicles was assayed after passive loading of vesicles in the presence of 10 mM α-amino[^{14}C]isobutyric acid [5]. When efflux was to be performed at a pH other than that in which the vesicles were originally pre-

pared, the vesicles were first centrifuged for 20 min (35 000 × *g*) and resuspended in the appropriate buffer: 100 mM potassium phosphate (pH 5.5)/10 mM $MgSO_4$; 100 mM potassium phosphate (pH 7.0)/10 mM $MgSO_4$; or 100 mM potassium carbonate (pH 9.0)/10 mM $MgSO_4$. The vesicles loaded with radioactive α-aminoisobutyric acid were usually divided into two suspensions, one of which was also loaded with 10 mM Na^+ (appropriate pH and salt) and the other with 10 mM K^+ (appropriate pH and salt). Efflux was initiated by a 1000-fold dilution of loaded vesicles into α-aminoisobutyric acid-free buffer, as described elsewhere [5].

Exchange was measured in the same manner except that the buffer into which vesicles were diluted to start the reaction contained 10 mM non-radioactive α-aminoisobutyric acid. Exchange thus differs from efflux in measuring outward translocations of solute under conditions in which the carrier is loaded with solute at all times. Efflux assays encompass movement of a loaded and unloaded carrier (return). Where indicated, efflux or exchange was assayed in the presence of a $\Delta\bar{\mu}_{H^+}$ that was generated by adding 20 mM potassium ascorbate and 2 mM TMPD to the dilution buffer.

Protein was determined by the method of Lowry et al. [31] using egg white lysozyme as the standard.

Results

α-Aminoisobutyric acid translocation in vesicles

Isolated membrane vesicles from wild-type *B. alcalophilus* exhibit α-aminoisobutyric acid uptake upon energization with ascorbate/TMPD at pH 9.0. As shown in Fig. 1, added Na^+ had a pronounced effect on this uptake, specifically upon the apparent K_m for α-aminoisobutyric acid. The K_m for α-aminoisobutyric acid was 80 μm at pH 9.0 in the absence, and 8.1 μM in the presence, of 10 mM Na^+. The addition of Na^+ also lowered the K_m for α-aminoisobutyric acid at pH 7.0, although not as dramatically (Table I). While the V for α-aminoisobutyric acid uptake by the wild type was unaffected by Na^+ at either pH, it was interesting that the V at pH 7.0 was greater than at pH 9.0. The non-alkalophilic mutant strain, KM23, exhibited no uptake of α-aminoisobutyric acid at pH 9.0. At pH 7.0, α-aminoisobutyric acid uptake was observed, and Na^+ was found to have

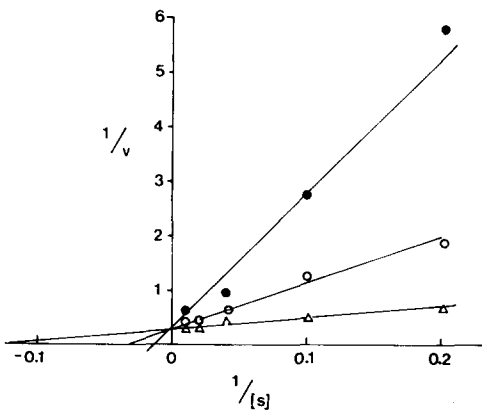

Fig. 1. A Lineweaver-Burk plot of α-aminoisobutyric acid uptake by vesicles from wild-type *B. alcalophilus*, showing the effect of Na^+. Uptake was assayed at pH 9.0, in 100 mM potassium carbonate buffer containing 10 mM $MgSO_4$, in the presence of no added Na^+ (•), 2 mM Na^+ (○), or 10 mM Na^+ (△).

no effect upon either the K_m or the V (Table I).

In previous studies [5] we had shown that passive α-aminoiosobutyric acid efflux from unenergized vesicles was Na^+-dependent in the wild-type, but not in KM23 vesicles. This was also true for efflux of L-malate and L-aspartate [5]. Exchange had not been examined at all, nor had efflux been studied as a function of pH. As shown in Fig. 2, the rates of α-aminoisobutyric acid efflux and exchange from unenergized wild-type vesicles were comparable, with efflux often just a bit faster. Thus, return of the unloaded carrier, or its real mechanistic equivalent, was not the rate-limiting step for solute efflux. Efflux and exchange were both markedly Na^+-dependent. Comparability of the rates of efflux and exchange (or slightly faster efflux) as well as Na^+-dependence was also observed in wild-type vesicles at pH 7.0 and pH 5.5 (Table II); the rates of efflux and exchange were, however, slightly lower at pH 7.0 and much lower at pH 5.5 than at pH 9.0. Efflux and exchange of α-aminoisobutyric acid in vesicles from KM23 also occurred at comparable rates, with efflux slightly faster, albeit with no dependence upon Na^+; this is shown at pH 7.0 in Fig. 3. The same pattern was observed, i.e., Na^+-independent efflux and exchange with the rate of efflux equal to or slightly greater than the rate of exchange, at pH 9.0 and pH 5.5 (Table II); the fastest

TABLE I

KINETIC PARAMETERS OF α-AMINOISOBUTYRIC ACID UPTAKE BY ENERGIZED VESICLES OF *B. ALCALOPHILUS* AND ITS NON-ALKALOPHILIC MUTANT DERIVATIVE

Membrane vesicles of *B. alcalophilus*, wild type and KM23, were prepared as described under Methods. The initial rates of uptake were measured at various concentrations of α-aminoisobutyric acid in the presence or absence of 10 mM Na^+. The results were plotted as $1/v$ vs. $1/S$ and the kinetic parameters were determined. In all experiments, both at pH 7.0 and pH 9.0, the amount of apparent uptake found in unenergized vesicles was subtracted. This value was no more than 10% of the total counts.

Strain	pH	Kinetic parameter			
		K_m (μM)		V (nmol/min per mg protein)	
		$-Na^+$	$+Na^+$	$-Na^+$	$+Na^+$
Wild type	9.0	80	8.1	3.6	3.6
	7.0	71	28	19	19
KM23	7.0	83	83	3.7	3.7

efflux and exchange in KM23 vesicles were observed at neutral pH. Thus, even in the Na^+-independent α-aminoisobutyric acid translocation catalyzed by KM23, return of the unloaded carrier is not rate-limiting, and the pH profile is consistent with a H^+-symport mechanism.

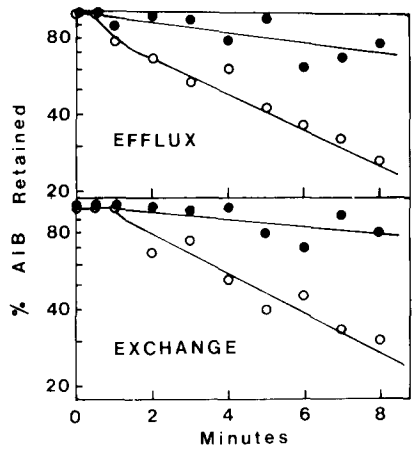

Fig. 2. The effect of Na^+ on α-aminoisobutyric acid efflux and exchange on wild-type *B. alcalophilus* vesicles at pH 9.0. Vesicles were loaded with α-amino[^{14}C]isobutyric acid in the presence (○) or absence (●) of 10 mM Na^+. Efflux or exchange was initiated by diluting 1000-fold into potassium carbonate buffer (○, plus Na^+; ●, minus Na^+) that was α-aminoisobutyric acid-free (efflux) or contained (exchange) 10 mM non-radioactive α-aminoisobutyric acid.

Establishment of a $\Delta\bar{\mu}_{H^+}$ by addition of ascorbate (20 mM) and TMPD (2 mM) strongly inhibited both efflux and exchange of α-aminoisobutyric acid in wild-type vesicles at pH 9.0 (Fig. 4), and pH 7.0 (Table III). These results suggest that the ternary complex between solute, coupling ion, and carrier is positively charged and hence inhibited by a $\Delta\bar{\mu}_{H^+}$, outside positive. Both processes were also inhibited

TABLE II

EFFECT OF Na^+ AND pH UPON EFFLUX AND EXCHANGE OF α-AMINOISOBUTYRIC ACID

Efflux and exchange were assayed, as described under Methods, by a 1000-fold dilution of α-amino[^{14}C]isobutyric acid-loaded vesicles into α-aminoisobutyric acid-free buffer (efflux) or buffer containing 10 mM non-radioactive α-aminoisobutyric acid. When present, Na^+ was added to the loading buffers as well as to the dilution buffers, which were at the indicated pH levels. The rates of efflux and exchange were expressed as $t_{1/2}$, or the time in which half of the α-aminoisobutyric acid was no longer retained by the vesicles.

Strain	Na^+ present (10 mM)	Rate of efflux ($t_{1/2}$)(min)			Rate of exchange ($t_{1/2}$)(min)		
		pH 5.5	pH 7.0	pH 9.0	pH 5.5	pH 7.0	pH 9.0
Wild type	+	11 ± 2.8	3.8 ± 0.6	3.5 ± 0.3	14 ± 7.9	5.5 ± 1.0	4.1 ± 0.3
	−	>15	>12	>10	>19	>12	>14
KM23	+	3.6 ± 0.4	2.4 ± 1.1	3.2 ± 0.9	4.2 ± 0.2	3.3 ± 0.9	4.2 ± 2.2
	−	3.6 ± 0.4	2.1 ± 0.3	4.2 ± 0.8	4.2 ± 0.2	3.3 ± 1.0	4.0 ± 1.0

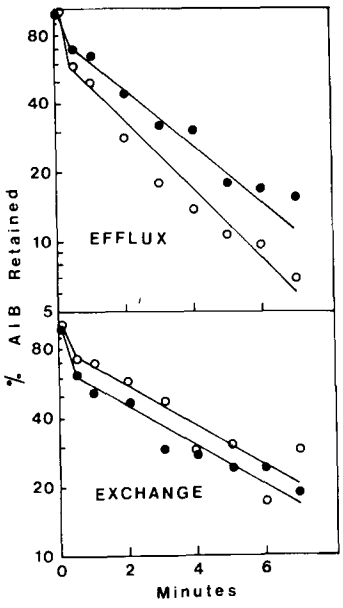

Fig. 3. The effect of Na^+ on α-aminoisobutyric acid efflux and exchange in KM23 vesicles at pH 7.0. Vesicles were loaded with α-amino[^{14}C]isobutyric acid at pH 7.0 in the presence (○) or absence (●) of 10 mM Na^+. Efflux and exchange were assayed as described under Methods and the legend to Fig. 2.

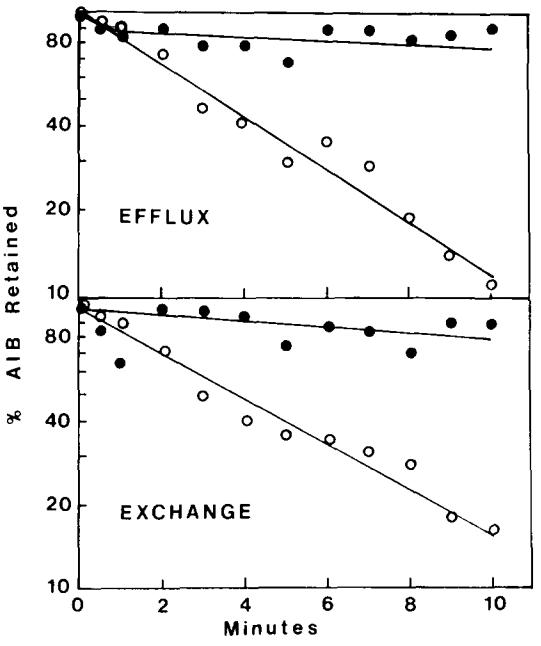

Fig. 4. The effect of a $\Delta\bar{\mu}_{H}^{+}$ upon α-aminoisobutyric acid efflux and exchange in wild-type *B. alcalophilus* vesicles at pH 9.0. Vesicles were loaded with α-amino[^{14}C]isobutyric acid at pH 9.0 in the presence of 10 mM Na^+. Efflux was initiated by 1000-fold dilution into potassium carbonate buffer, pH 9.0, containing 10 mM Na^+ either with (●) or without (○) 20 mM potassium ascorbate and 2 mM TMPD. Exchange was initiated in the same manner, with (●) or without (○) ascorbate/TMPD, except that the diluting buffer contained 10 mM non-radioactive α-aminoisobutyric acid as well as 10 mM Na^+.

by a $\Delta\bar{\mu}_{H}^{+}$ in KM23 vesicles at pH 7.0. (Table III). The latter vesicles were not assayed at pH 9.0 because no- substantial $\Delta\bar{\mu}_{H}^{+}$ was established with complete reproducibility at that pH. The effects of ascorbate/TMPD in these experiments were completely abolished when 10 μM gramicidin was also included in the dilution buffer (data not shown).

Mutational loss of L-*methionine transport*

Strain MM7 was an alkalophilic mutant selected for ethionine-resistance and shown to be deficient in

TABLE III

EFFECT OF $\Delta\bar{\mu}_{H}^{+}$ UPON α-AMINOISOBUTYRIC ACID EFFLUX

The experiments were conducted as described in the legend to Table II, except that 10 mM Na^+ was present in all the wild-type vesicle suspensions, and, where indicated, potassium ascorbate (20 mM) and TMPD (2 mM) were added to the dilution buffer.

Strain	pH	Rate of efflux ($t_{1/2}$)(min)		Rate of exchange ($t_{1/2}$)(min)	
		−ASC/TMPD	+ASC/TMPD	−ASC/TMPD	+ASC/TMPD
WT	9.0	3.5 ± 0.3	>7	4.1 ± 0.3	>10
	7.0	3.8 ± 0.6	>7	5.5 ± 1.0	>10
KM23	7.0	2.1 ± 0.3	>6	3.3 ± 1.0	>7

L-methionine transport. If the proton-coupled porters in non-alkalophilic strains are a genetically distinct set of porters from the Na^+-coupled porters of the alkalophilic parent, then non-alkalophilic derivatives of MM7 should exhibit L-methionine transport comparable to that seen in other non-alkalophilic strains such as KM23. If the porters used are the same in wild type and KM23, and only the coupling ion is changed upon mutation, then at least some non-alkalophilic derivatives of MM7 should retain the deficiency in L-methionine transport. Non-alkalophilic derivatives of MM7 were selected after mutagenesis and plating on solidified L-malate-containing medium at pH 6.8. It should be noted that growth of transport-positive revertants of the MM7 was slightly favored, since DL-ethionine was absent and small amounts of L-methionine were present in the yeast extract in the medium. Nevertheless, as shown in Table IV, most of the nine non-alkalophilic strains assayed at random exhibited significantly reduced levels of L-methionine transport when compared to KM23. Importantly, the ratio of L-methionine transport activity of at least two MM7NA strains to that of KM23 was as low as the ratio of transport in MM7 to the wild type. None of the non-alkalophilic derivatives of MM7 showed Na^+-dependent L-methionine uptake.

TABLE IV

THE EFFECT OF Na^+ ON L-METHIONINE UPTAKE BY CELLS OF WILD-TYPE *B. ALCALOPHILUS*, MM7 AND THEIR NON-ALKALOPHILIC DERIVATIVES

Uptake of L-methionine by washed cell suspensions was assayed in 25 mM potassium phosphate buffer (pH 7.0) to which 10 mM L-malate and either 10 mM KCl or 10 mM NaCl were added. Mutant strains designated MM7NA1–9 represent non-alkalophilic derivatives of MM7.

Strain	L-Methionine uptake (nmol/min per mg protein)	
	$-Na^+$	$+Na^+$
Wild type	0.51	1.25
KM23	3.33	3.33
MM7	0.15	0.29
MM7NA1	2.38	2.38
MM7NA2	0.62	0.62
MM7NA3	1.05	1.05
MM7NA4	0.64	0.64
MM7NA5	1.09	1.09
MM7NA6	1.04	1.04
MM7NA7	2.27	2.27
MM7NA8	1.31	1.31
MM7NA9	3.23	3.23

The retention of the L-methionine transport mutation upon further mutation of MM7 to non-alkalophily supported the view that the porters that function in a proton-coupled manner in non-alkalophiles are the same porters as those in alkalophiles except for loss of Na^+-coupling. To bypass the reversion problem, however, this question was addressed in an additional determination. The frequency of spontaneous mutation of wild-type *B. alcolophilus* to non-alkalophily had previously been found to be one in $1.6 \cdot 10^9$cells [25]. In the current study, the frequency of mutation of MM7 to non-alkalophily was determined with 5 mM DL-ethionine present in all the solutions and media. Were non-alkalophilic derivatives to possess a distinct L-methionine transport system, the frequency of mutation to non-alkalophily and DL-ethionine-resistance would encompass two separate muational events and would be much lower than that seen for the wild type. On the contrary, the frequency found for MM7 was one non-alkalophile for every 10^8 cells, again supporting the idea that the porters in the non-alkalophiles are the same as the porters in the alkalophilic parent except for the change in coupling ion.

Discussion

Na^+-coupled translocation of α-aminoisobutyric acid in isolated membrane vesicles from wild-type *B. alcalophilus* exhibited many similarities to Na^+-coupled symporters from other bacteria. As has been shown in other alkalophiles [2] as well as enteric bacteria [15–17,31], the effect of Na^+ was to reduce the K_m, with no effect on the V. On the other hand, no appreciable rate of α-aminoisobutyric acid uptake (with or without Na^+) was observed unless an electron donor was provided to generate a $\Delta\bar{\mu}_{H^+}$. The Na^+/α-aminoisobutyric acid symport system in the alkalophile further resembled the Na^+/melibiose symport system [31] with respect to the characteristics of passive solute efflux and exchange in membrane vesicles. First, exchange was no faster than efflux, indicating that return of the unloaded carrier from the

outside to the inside was not the rate-limiting step for efflux. While this was also true of Na^+/melibiose symport in *E. coli* vesicles [31], the converse was true for H^+-coupled lactose transport in *E. coli* vesicles [29]. The observation that in *B. alcalophilus* vesicles, exchange is consistently a little slower than efflux siggests that in this system the unloaded carrier may actually move more quickly than the loaded carrier in the absence of energy. A second similarity between Na^+/α-aminoisobutyric acid symport in *B. alcalophilus* vesicles and Na^+/melibiose symport in *E. coli* [31] vesicles was that a $\Delta\bar{\mu}_{H^+}$ (outside positive) inhibited both efflux and exchange of solute. This suggests that the ternary complex between porter, coupling ion and solute may be positively charged in these systems, whereas the ternary complex in the H^+/lactose symport system appears to be electrically neutral [30].

Most interesting was the observation that α-aminoisobutyric acid translocation in KM23, apparently H^+-coupled [23], retained the same properties as the wild-type Na^+/α-aminoisobutyric acid symport with respect to the rates of efflux vs. exchange and the effect of a $\Delta\bar{\mu}_{H^+}$ on these processes. On the basis of the determinations on the L-methionine transport-deficient strain MM7, we conclude that the porters operating in the non-alkalophile are the same ones that function in the wild type except that the alteration in ion-coupling occurred. Previously, we proposed as a working model, that Na^+/solute symporters may possess a common Na^+-translocating subunit that is also part of the Na^+/H^+ antiporter [23,5]; this subunit could be the site of mutation in the non-alkalophilic KM strains. If so, the porters may have the inherent capacity to translocate solutes in symport with protons. In the wild type, this capacity is not expressed (even in vesicles at pH 7.0 and 5.5), perhaps because the Na^+-translocating element blocks or inhibits proton utilization. Mutational loss of the Na^+-translocating capacity allows expression of the latent H^+/solute symport, whose general properties remain similar except for the ion used. This model is speculative, and is only one of the logical possibilities, although it is consistent with all the data to date. It is also consistent with the findings of Zilberstein et al. [32], who have found very similar pleiotropic mutational losses of Na^+-coupled porter functions in *E. coli*. A definitive test of the model awaits structural determinations.

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