

Patterns of Electrochemical Proton Gradient Formation by Membrane Vesicles from an Obligately Acidophilic Bacterium

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Isolated membrane vesicles from the obligately acidophilic bacterium *Bacillus acidocaldarius* generated an electrochemical gradient of protons ($\Delta\bar{\mu}_{H^+}$) upon energization with ascorbate-phenazine methosulfate at pH 6.0 or 3.0. At pH 6.0, there was little or no transmembrane pH gradient (ΔpH), but a transmembrane electrical potential ($\Delta\psi$) of ca. -77 mV, positive out, was observed. At pH 3.0, a ΔpH equivalent to -100 mV, acid out, and a $\Delta\psi$ of -73 mV, positive out, were observed upon energization. The total magnitude of the $\Delta\bar{\mu}_{H^+}$ was higher than that of whole cells at acid pH, but the very large ΔpH s and the reversed $\Delta\psi$ s, i.e., inside positive, that are typical of acidophile cells were not observed in the vesicles. The vesicles exhibited energy-dependent accumulation of α -aminoisobutyric acid that was inhibited by both nigericin and valinomycin (plus K^+) at pH 3.0 but was inhibited little by nigericin at pH 6.0.

Obligately acidophilic bacteria maintain a cytoplasmic pH of 6.0 or higher while growing at external pH values as low as 2 (2, 12). There is general agreement that establishment of this enormous transmembrane pH gradient (ΔpH) is energy dependent and that its maintenance is predicated upon the existence of a transmembrane electrical potential ($\Delta\psi$) that is positive inside, i.e., the reverse of the usual direction. Such a reversed $\Delta\psi$ has been found in all of the acidophilic species examined (2, 12); in both *Bacillus acidocaldarius* (11) and *Thiobacillus acidophilus* (16), abolition of the $\Delta\psi$ resulted in collapse of the ΔpH . Beyond the consensus on the orientation of the $\Delta\psi$, however, there is uncertainty and controversy with respect to the bioenergetic properties of acidophiles. Among the uncertainties is the chemical nature and origin of the $\Delta\psi$ (e.g., references 8, 16, and 19). We undertook a study of isolated membrane vesicles from *B. acidocaldarius* to determine whether the pattern of the electrochemical proton gradient ($\Delta\bar{\mu}_{H^+}$ that is typical of acidophiles, i.e., very large ΔpH and reversed $\Delta\psi$, is formed and can be examined in such an in vitro system. If not, the $\Delta\bar{\mu}_{H^+}$ of vesicles might reflect a partial set of the normal ion movements, providing the opportunity to study the primary ion fluxes and, possibly, to reconstitute the whole cell pattern. The $\Delta\bar{\mu}_{H^+}$ patterns of a vesicle system from acidophiles have not previously been reported.

MATERIALS AND METHODS

Organism and growth conditions. *B. acidocaldarius* (ATCC 27009) was grown with shaking at 50°C in a basal salt medium (11) adjusted to pH 3.5 with H_2SO_4 . Potassium (D,L)-malate was added from a separate sterile solution to a final concentration of 50 mM. Growth was followed turbidometrically with a Klett-Summerson colorimeter (no. 42 filter).

Preparation and characterization of right-side-out vesicles. Care was taken to harvest only mid-logarithmic-phase cells when preparing membrane vesicles by a modification of the lysozyme method of Kaback (9). Preliminary experiments had indicated that only actively growing cells were susceptible to lysozyme digestion of their cell walls; thus, cells harvested at room temperature at $10,000 \times g$ for 10 min were

immediately suspended in prewarmed (50°C) basal salt medium (with 50 mM potassium phosphate)—50 mM (D,L)-malate—10% (wt/vol) mannitol—1 mg of lysozyme per ml adjusted to pH 6.0. Previous work has shown that pH 6.0 is a typical cytoplasmic pH for cells of *B. acidocaldarius* (11), and that equilibration of cells at that pH failed to significantly compromise the viability of the cells when they were returned to acid pH (5). The protease inhibitors phenylmethylsulfonyl fluoride, *p*-toluene sulfonylic acid, and *p*-toluene sulfonyl fluoride were added to 1 mM as suggested by Heefner and Harold (7). After incubation at 50°C for ca. 15 min, protoplasts formed. The protoplasts were harvested by centrifugation at $15,000 \times g$ for 30 min. Homogenized protoplasts were then rapidly diluted into 100 volumes of 50 mM potassium phosphate or sodium phosphate (pH 3.0 or 6.0) plus 10 mM $MgSO_4$ at 50°C in the presence of DNase (10 $\mu g/ml$). Vesicles were harvested at room temperature by centrifugation for 30 min at $35,000 \times g$. Whole cells and large debris were separated from membrane vesicles by differential centrifugation of resuspended vesicles at low speed. Vesicles were subsequently washed several times. Thin-section electron micrographs of the membrane vesicle preparations revealed circular closed sacs, surrounded by a unilamellar membrane. The purified vesicles contained less than 2% of the cellular DNA as determined by the diphenylamine assay of Burton (1) and less than 1% of the cellular isocitrate dehydrogenase as determined by the method of Daron et al. (3). The vesicle volume was calculated to be 2.6 μl of intravesicular water per mg of vesicle protein, using tritiated water and [^{14}C]sorbitol as markers (20). The preparations were relatively uniform in vesicle size and were predominantly right-side-out as judged by the failure of NADH to energize solute transport or by generation of a $\Delta\bar{\mu}_{H^+}$, whereas NADH was an effective electron donor for everted preparations (data not shown).

Dithionite-reduced minus ferricyanide-oxidized difference spectra were recorded at room temperature with a Perkin-Elmer 557 dual beam spectrophotometer; a typical spectrum of the vesicles is shown in Fig. 1. The following wavelength pairs and extinction coefficients were used to estimate the amounts of the apparent cytochrome *b* species and the apparent *a*-type cytochromes: cytochrome *b*, $A_{558-575}$, $E_{17.5}$ (4); and cytochrome *a*, $A_{605-630}$, $E_{20.5}$ (21). The *a*-type

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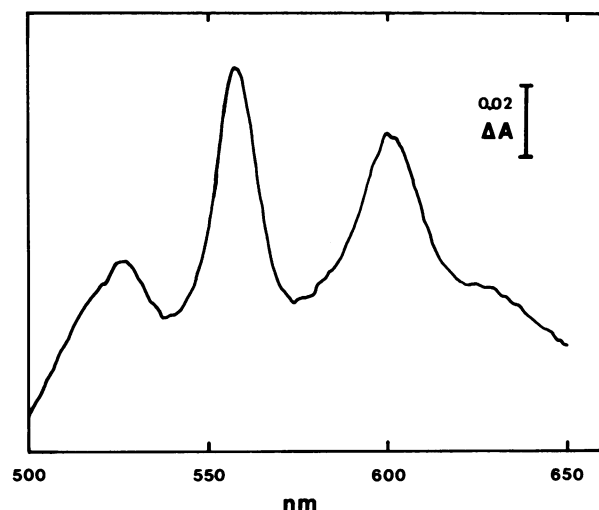


FIG. 1. Difference spectrum of vesicle membranes from *B. acidocaldarius*. Dithionite-reduced minus ferricyanide-oxidized samples of membrane vesicles (2.7 mg of protein per ml) were recorded at room temperature as described in the text.

cytochrome content of membrane vesicles was calculated to be 0.8 nmol/mg of protein, and the cytochrome *b* content was 0.53 nmol/mg of protein. The lack of an obvious peak or shoulder in the region expected for cytochrome *c* (e.g., 550 nm) is notable and has recently been described by others (4a).

Measurements of the ΔpH and $\Delta\psi$. The pH was determined from the distribution of a weak acid, [7- ^{14}C]benzoic acid (25 mCi/mmol) or DMO (5,5-dimethyl-[2- ^{14}C]oxazolindione-2,4-dione) (8.8 mCi/mmol) in the flow dialysis assay of Ramos et al. (17) as described by Mandel et al. (14). The buffer pumped through the lower chamber was prewarmed so that the temperature in the chamber was 50°C. Experiments were performed at either pH 3.0 or 6.0 with 50 mM potassium or sodium phosphate plus 10 mM $MgSO_4$ adjusted to the appropriate pH. Radiolabeled benzoic acid (55 μM) or DMO (43 μM) was added to 800 μl of vesicles (ca. 8 mg of vesicle protein) in the upper chamber, 1.8-ml fractions were collected, and 1-ml samples were assayed for radioactivity by liquid scintillation counting. The ΔpH was calculated by the formula of Waddell and Butler (22).

The $\Delta\psi$ was similarly measured in a flow dialysis assay at pH 3.0 or 6.0 by following the distribution of 3.6 μM [3H]tetraphenylphosphonium (TPP^+) (5.6 Ci/mmol) or 400 μM [^{14}C]thiocyanate (SCN^-) (1.6 mCi/mmol). In some experiments the distribution of 200 μM ^{86}Rb (2 mCi/mmol) in the presence of 10 μM valinomycin was used to measure the $\Delta\psi$. For such measurements the vesicles were prepared in sodium phosphate buffer, containing 10 mM $MgSO_4$, taking care to exclude potassium from the preparation. The $\Delta\psi$ was also measured in a filtration assay (18) at 50°C with using 10 μM [3H]TPP $^+$ (500 mCi/mmol). Steady-state values of TPP^+ , Rb^+ , or SCN^- uptake were used to calculate the $\Delta\psi$ from the Nernst equation.

Measurement of α -AIB transport. Uptake of radioactive aminoisobutyric acid (AIB) was measured by a filtration assay as described previously (6). The reaction mixture contained 50 mM potassium phosphate (pH 3.0 or 6.0) and 10 mM $MgSO_4$. Vesicles were added to 1 mg of protein per ml and incubated at 50°C with constant oxygenation. Potassium ascorbate and phenazine methosulfate (PMS) were added to final concentrations of 20 and 0.2 mM, respectively. After 1

min of incubation in the presence of ascorbate-PMS plus any indicated inhibitors, the reaction was started by the addition of 40 μM [^{14}C]AIB (4.5 mCi/mmol). Samples (100 μl) were removed at various times, filtered through nitrocellulose filters (pore diameter, 0.45 μm), and washed at room temperature with a 10-fold excess of buffer. Radioactivity was determined by liquid scintillation spectrometry.

Chemicals. Valinomycin, lysozyme, carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP), and DNase I were from Sigma Chemical Co., St. Louis, Mo. [7- ^{14}C]benzoic acid (25 mCi/mmol), [1- ^{14}C]AIB (4.5 mCi/mmol), [2- ^{14}C]DMO (8.8 mCi/mmol), [^{14}C]SCN $^-$ (1.6 mCi/mmol), and ^{86}Rb (2 mCi/mmol) were purchased from New England Nuclear Corp., Boston, Mass. [3H]TPP $^+$ (5.6 Ci/mmol) was a product of Nuclear Research Center, Negev, Israel.

RESULTS

$\Delta\bar{\mu}_H$ patterns at pH 6.0. The cytoplasmic pH of *B. acidocaldarius* cells has been found to remain stable at close to pH 6.0 over a very broad range of external pH values (11). Therefore, a series of $\Delta\bar{\mu}_H$ determinations was first conducted using vesicles that were equilibrated at pH 6.0. Upon addition of ascorbate-PMS to such vesicles, most preparations exhibited no uptake of weak acids (benzoic acid or DMO) or of the weak base methylamine (data not shown), suggesting that at pH 6.0, $\Delta pH = 0$. Occasionally, a preparation showed a little uptake of weak acids upon energization. A $\Delta\psi$, positive out, was observed upon energization of the vesicles (Fig. 2). After addition of ascorbate-PMS at pH 6.0 uptake of the lipophilic cation TPP^+ occurred (Fig. 2A). Calculations of the $\Delta\psi$ from such flow dialysis assays of TPP^+ uptake yielded values of ca. -70 mV; experiments with a filtration assay for TPP^+ uptake at pH 6.0 yielded a similar value of -77 mV for the $\Delta\psi$. Formation of a $\Delta\psi$, positive out, was also indicated by energy-dependent uptake of $^{86}Rb^+$ in the presence of valinomycin and by an apparent exclusion of the lipophilic anion SCN^- (Fig. 2B and C). The effects of energization on SCN^- distribution were delayed in time relative to TPP^+ or $^{86}Rb^+$ uptake. Calculation of the $\Delta\psi$ from the experiments with valinomycin-mediated $^{86}Rb^+$ uptake gave a value of -50 mV. Since the TPP^+ uptake was unchanged by substituting Na^+ for K^+ in the buffer, the difference in buffers used in the protocols does not account for the different $\Delta\psi$ values with TPP^+ versus those with $^{86}Rb^+$. No effect upon either the ΔpH or the $\Delta\psi$ was observed when the experiments were conducted in the growth medium salts (adjusted to pH 6.0) instead of potassium phosphate buffer.

$\Delta\bar{\mu}_H$ patterns at pH 3.0. The ΔpH and $\Delta\psi$ were then assayed upon addition of ascorbate-PMS to *B. acidocaldarius* vesicles that had been equilibrated with buffer at pH 3.0. Upon addition of the electron donor, benzoic acid was taken up, indicating the presence of a ΔpH , acid out (Fig. 3A). Upon subsequent addition of CCCP to a final concentration of 10 μM , efflux of the accumulated benzoic acid was observed. When either 10 μM CCCP (Fig. 3B) or 10 mM potassium thiocyanate (Fig. 3C) was added before the electron donor, essentially no benzoic acid accumulation occurred after addition of ascorbate-PMS. In determinations with many independent preparations, the pH of the intravesicular space rose to 4.4 to 4.9 upon addition of ascorbate-PMS, with a typical value of pH 4.8. This was equivalent to -100 mV, a substantial ΔpH , but not nearly as large as that (ca. -180 mV) exhibited by whole cells at pH 3.0.

Also, in contrast to whole cells at acidic external pH values, the $\Delta\psi$ of energized vesicles at pH 3.0 was positive

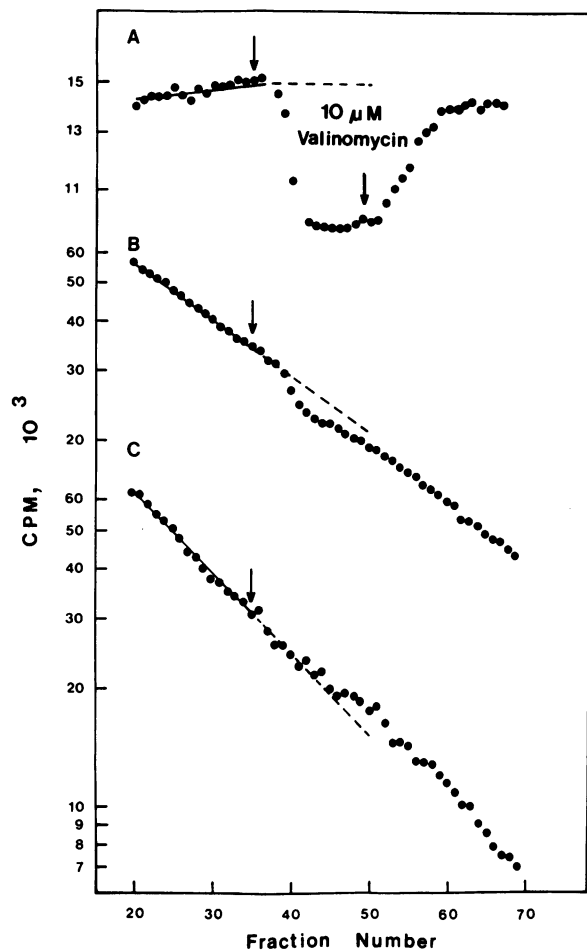


FIG. 2. Generation of a $\Delta\psi$ by *B. acidocaldarius* vesicles at pH 6.0. Membrane vesicles were suspended in potassium phosphate buffer (curves A and C) or sodium phosphate buffer (curve B) at pH 6.0. The $\Delta\psi$ was determined by flow dialysis as described in the text. Vesicles were added to the upper chamber at a concentration of 8 mg of protein per ml. Where indicated by the arrow, 20 mM potassium ascorbate (A and C) or sodium ascorbate (B) and 0.1 mM PMS was added as an energy source. Curve A is the flow dialysis pattern of [^3H]TPP $^+$ (3.6 μM , 5.6 Ci/mmol) uptake to which 10 μM valinomycin was added at the point indicated by the second arrow. Curve B is the pattern for $^{86}\text{Rb}^+$ (200 μM , 2 mCi/mmol) uptake in the presence of 10 μM valinomycin. Curve C is the pattern for [^{14}C]SCN $^-$ (400 μM , 1.6 mCi/mmol) uptake.

out. Upon addition of ascorbate-PMS, vesicles accumulated TPP $^+$ (Fig. 4A), although in some experiments TPP $^+$ appeared to be briefly excluded before its accumulation. Energized vesicles, at pH 3.0, also accumulated $^{86}\text{Rb}^+$ in the presence of valinomycin (Fig. 4B) and appeared to exclude SCN $^-$ (Fig. 4C). Calculations from the TPP $^+$ and $^{86}\text{Rb}^+$ uptake data in Fig. 4 indicated the formation of a $\Delta\psi$ of -73 mV and -50 mV, respectively. Again, the level of TPP $^+$ uptake was the same in sodium phosphate buffer as it was in potassium phosphate buffer, so the lower value obtained with $^{86}\text{Rb}^+$ cannot simply be explained by the absence of K $^+$. The difference between the rates of appearance of the various probes in the dialysate before energization does not appear to have any significance; this is observed with other probes and seems to relate to binding properties of individual agents. Filtration assays of TPP $^+$ uptake resulted in values similar to those found by flow dialysis; TPP $^+$ uptake was

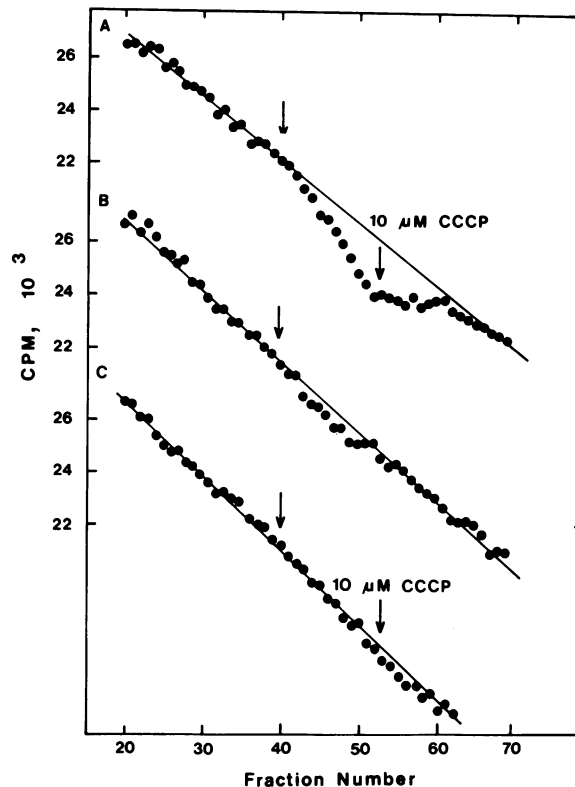


FIG. 3. CCCP- and cyanide-sensitive generation of a ΔpH by energized vesicles from *B. acidocaldarius*. Membrane vesicles from *B. acidocaldarius* were suspended in pH 3.0 buffer (potassium phosphate) as described in the text. The distribution of [^{14}C]benzoic acid (42 μM , 25 mCi/mmol) was assayed by flow dialysis. Vesicles were added to the upper chamber at a concentration of 8 mg of protein per ml. At the fraction indicated by the left-hand arrows, 20 mM potassium ascorbate and 0.1 mM PMS were added as an energy source. Curve A is an experiment in which 10 μM CCCP was added after energization at the time indicated by the second arrow. In curve B, 10 μM CCCP was added at fraction zero before energization with ascorbate-PMS. In curve C, 10 mM potassium thiocyanate was added at fraction zero before ascorbate-PMS and later followed by the addition of 10 μM CCCP at the second arrow.

inhibited 83 to 95% by the inclusion of gramicidin, valinomycin, or CCCP (at 10 μM each) in the incubation mixture (data not shown).

Since the above determinations were conducted in potassium or sodium phosphate buffer containing MgSO_4 , it was possible that some absent ion was required for establishment of a typical $\Delta\psi$ of an acidophile, positive in, rather than the conventional $\Delta\bar{\mu}_{\text{H}^+}$, acid and positive out, of ca. -173 mV was still observed when the experiments were conducted in complete growth medium salts. The addition of Na $^+$ or Cl $^-$ to the equilibration buffer also failed to affect the $\Delta\bar{\mu}_{\text{H}^+}$.

AIB uptake by energized vesicles. Upon addition of ascorbate-PMS, vesicles from *B. acidocaldarius* accumulated AIB; addition of NADH did not result in AIB uptake. The AIB accumulation was linear with time for at least 2 min (data not shown). AIB uptake in potassium phosphate buffer was very sensitive to inhibition by nigericin, CCCP, and valinomycin at pH 3.0 (Table 1). At pH 6.0, nigericin had little effect, but CCCP and valinomycin strongly inhibited AIB uptake. Notably, although the magnitude of the $\Delta\bar{\mu}_{\text{H}^+}$ was quite different at the two pH values examined, the rate

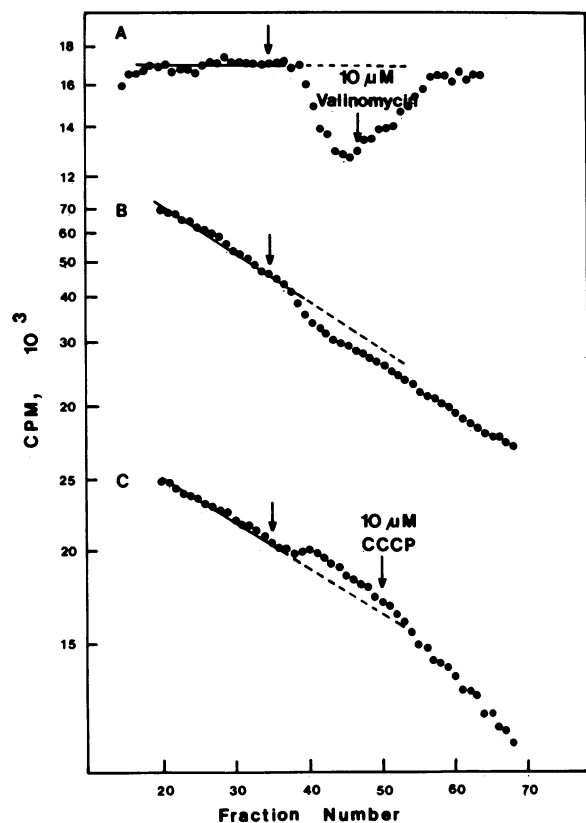


FIG. 4. Generation of a $\Delta\psi$ by *B. acidocaldarius* vesicles at pH 3.0. Conditions were the same as described in the legend to Fig. 2, except that the pH was 3.0 and the specific activity of [14 C]SCN (curve C) was 0.5 mCi/mmol instead of 1.6 mCi/mmol.

of AIB uptake was quite similar. Also, when vesicles were allowed to accumulate AIB to steady-state, the $\Delta\bar{\mu}_{AIB}$ was -75 mV at pH 6.0 and -72 mV at pH 3.0. No effect of added Na^+ on the rate or extent of AIB uptake was observed.

DISCUSSION

The establishment of a $\Delta\bar{\mu}_{H^+}$ upon addition of an electron donor to vesicles from *B. acidocaldarius* indicates that the respiratory chain of this obligately acidophilic bacterium possesses the expected ability to extrude protons. Notably, however, the $\Delta\bar{\mu}_{H^+}$ pattern of the vesicles, as a function of external pH, resembles that of neutralophilic bacteria except that it is shifted downward on the pH scale. For example, energized vesicles from *Escherichia coli* exhibit both a ΔpH and a $\Delta\psi$ (acid and positive out, respectively) at pH values from 5.5 to 7, but at moderately alkaline pH values, the $\Delta\psi$ is the sole component of the $\Delta\bar{\mu}_{H^+}$ (10). Vesicles from *B. acidocaldarius* similarly exhibit both a ΔpH and $\Delta\psi$ of the same orientation as in *E. coli* at a relatively acid pH value, which in this case is pH 3.0. At an external pH that is still acidic, pH 6.0, the acidophile vesicles exhibit only a $\Delta\psi$. The absence of a ΔpH at pH 6.0 may, in fact, reflect the presence of antiport activities that are active at more acidic pH values than in neutralophilic organisms. In addition to the similar, albeit pH-shifted patterns of the $\Delta\bar{\mu}_{H^+}$ components, the magnitude of the ΔpH and $\Delta\psi$ are comparable to those found in nonacidophiles (13). The total $\Delta\bar{\mu}_{H^+}$ generated by vesicles at very acid pH is higher than that observed in whole cells at comparable pH values (11) since a conventional $\Delta\psi$ rather

TABLE 1. The uptake of AIB by membrane vesicles from *B. acidocaldarius*^a

Addition	AIB uptake (nmol/min per mg) at:	
	pH 3.0	pH 6.0
None	0.27	0.22
Ascorbate-PMS	1.09	1.11
Ascorbate-PMS, 1 μ M nigericin	0.16	0.88
Ascorbate-PMS, 10 μ M CCCP	0.13	0.13
Ascorbate-PMS, 10 μ M valinomycin	0.12	0.15

^a Membrane vesicles were incubated in 50 mM potassium phosphate (pH 3.0 or 6.0) containing 10 mM $MgSO_4$. Ascorbate (20 mM), PMS (0.2 mM), and inhibitors, when present, were added 1 min before the addition of 40 μ M [14 C]AIB. After 1 more min, samples were filtered, washed, and counted as described in the text.

than the reversed $\Delta\psi$ is formed. The establishment of such a substantial $\Delta\bar{\mu}_{H^+}$ and the capacity for concentrative solute uptake indicate that vesicles from acidophiles possess an integrity that is at least comparable to other bacterial vesicles. The constancy of the $\Delta\psi$ over a broad pH range is typical of vesicle systems (10, 13).

The patterns of inhibition of AIB uptake are consistent with the $\Delta\bar{\mu}_{H^+}$ patterns found. That is, at pH 3.0, solute accumulation was markedly inhibited by inhibitors that abolish the ΔpH (nigericin), the $\Delta\psi$ (valinomycin in a K^+ -containing medium), or both (CCCP). At pH 6.0, nigericin had little effect, whereas those inhibitors that reduce the $\Delta\psi$ inhibited AIB uptake. It is interesting that a transport system that exists in cells which do not generate a $\Delta\psi$, positive out, should show coupling to such a $\Delta\psi$. Presumably, an H^+ -AIB symport energizes the translocation, at least at pHs at which AIB is neutral, and has the capacity to utilize a $\Delta\psi$, positive out. It is intriguing that the $\Delta\bar{\mu}_{AIB}$ values calculated from steady-state levels of accumulation were -75 mV at pH 6.0, at which the $\Delta\bar{\mu}_{H^+}$ (all $\Delta\psi$) was -77 mV, and -72 mV at pH 3.0, at which the $\Delta\bar{\mu}_{H^+}$ was -173 mV. It will be of interest to study the details of solute transport in this vesicle system.

The extremely large ΔpH that acidophile cells exhibit at very acidic pH (2, 12) was not observed in energized vesicles at pH 3.0; nor was the reversed $\Delta\psi$ that is found in acidophile cells (2, 12) and spheroplasts (15) at very acid pH observed in energized vesicles at pH 3.0. In view of findings with whole cells (11, 16), it is reasonable to speculate that the reversed $\Delta\psi$ would be necessary for the maintenance and even perhaps for the generation of the larger ΔpH s seen in cells. Thus, a fundamental acidophile property, the establishment of a $\Delta\psi$, positive in, may not be mediated by some simple secondary porter. It will be of interest to establish an everted vesicle system which might facilitate attempts to reconstitute the factors required for the formation of the reversed $\Delta\psi$. Further studies of the right-side-out vesicles will be directed at properties of primary proton pumping, antiporters, and mechanisms of solute transport.

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