JOURNAL OF BACTERIOLOGY, Dec. 1983, p. 1352–1355
0021-9193/83/121352-04$02.00/0

Vol. 156, No. 3

Cytoplasmic pH Homeostasis in an Acidophilic Bacterium, *Thiobacillus acidophilus*

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Received 11 July 1983/Accepted 20 September 1983

The cytoplasmic buffering capacity of *Thiobacillus acidophilus* (along with membrane properties) is responsible for the cytoplasmic pH homeostasis in metabolically compromised cells. When a large influx of H^+ occurs, the cytoplasmic buffering capacity prevents drastic changes in pH; in addition, this influx, by increasing the positive membrane potential, eventually leads to a cessation of further H^+ influx.

We previously reported (5, 6) that the respiring cells of *Thiobacillus acidophilus* (optimal pH, ~3) possess a near-neutral cytoplasmic pH (ΔpH, ~3) and a membrane potential ($\Delta\Psi$) of ~+70 mV, i.e., of a polarity reversed from that found in neutrophilic bacteria; thus, these bacteria possess a proton motive force (Δp) of ~−110 mV. Even though a large ΔpH exists, the cytoplasmic pH in this bacterium is a remarkably stable parameter. It is affected only minimally (~1-U decrease) by a complete inhibition of respiration, even when the ATPase inhibitor dicyclohexylcarbodiimide is also present (10); by treatment of cells with protonophores (6); or by the prolonged starvation of cells, even to a point of virtual elimination of culture viability (10).

All of the above treatments cause an increase in $\Delta\Psi$ to +100 mV or even higher, so that Δp is decreased to very low values (~−30 mV). Thus, at optimal external pH values, the ΔpH maintenance in this bacterium under nonmetabolizing conditions essentially represents an equilibrium situation in which $\Delta\Psi$ and ΔpH of opposite polarity nearly balance each other. Nothing is known about the nature of the positive $\Delta\Psi$. Hsung and Haug (2), who studied the acidophile *Thermoplasma acidophila*, postulated that it represented a Donnan potential of charged cellular macromolecules, which by undergoing appropriate fluctuations presumably kept H^+ out of the cells. We previously proposed that the increase in $\Delta\Psi$ observed under metabolically inert conditions was due to a net influx of H^+ into the cells (6, 10).

In an attempt to shed some light on the matter, we measured H^+ fluxes in *T. acidophilus* cells under conditions of active metabolism as well as in cells treated with various inhibitors and ionophores. These measurements showed that metabolically inert conditions trigger a large H^+ influx into the cells. Since the H^+ influx only minimally affects the cytoplasmic pH, the results suggested a strong buffering capacity in the cytoplasm; consequently, we quantified this capacity.

(A preliminary report of this work has been presented [XIII Int. Congr. Microbiol., Boston, Mass., 8–13 August 1982, abstr. no. P44:3].)

T. acidophilus was grown heterotrophically at pH 3.0 in a mineral salts-glucose medium as previously described (6). The cells were washed once in deionized water adjusted to pH 3.0 by the addition of H_2SO_4 and suspended in water at the same pH to a density of 5 mg of protein per ml. Ten milliliters of this cell suspension was placed in an electrode reaction chamber (Brinkmann Instruments, Inc., Westbury, N.Y.) which was maintained at 29°C, stirred with a magnetic flea, and aerated by a constant stream of O_2. Changes in the external pH of this suspension were monitored using a combination electrode and a model 135 pH meter (Corning Glass Works, Medfield, Mass.) connected to a model 8371-20 recorder (Cole Parmer, Chicago, Ill.). The recording of pH was begun after the cells had been stirred in the chamber for 15 min. The pH was monitored for several minutes before the addition of various agents. Parallel control measurements were always run to ensure that the various agents themselves did not alter the external pH.

To determine the buffering capacity in disrupted cells, the following procedure was used. Cells were washed in deionized water and suspended to a density of 5 mg of protein per ml in water containing 5 or 10% Triton X-100 (Sigma Chemical Co., St. Louis, Mo.). Ten milliliters of this suspension was transferred to the electrode chamber at 29°C and 20-μl aliquots of 0.05 M HCl were added while the pH was recorded until it was decreased from 6 to 5. The buffering

capacity was expressed as the amount of H^+ needed per milligram of protein to bring about this decrease. Parallel control measurements were made using (i) water alone, (ii) intact cells suspended in water, and (iii) the Triton solution of appropriate concentration, and the results were corrected by subtracting the small buffering capacity of the intact cells and the Triton solution from the buffering capacity of the extract.

Protein was determined by the method of Lowry et al. (3). Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and valinomycin were purchased from Sigma, and nigericin was purchased from Calbiochem Behring Corp., La Jolla, Calif.

At their optimal pH (i.e., 3.0), fresh cell suspensions of *T. acidophilus* incubated with stirring under a stream of oxygen exhibited no change in the external pH; but when respiration was inhibited either by anaerobiosis or by the addition of azide or cyanide (10 mM [6]), there was a marked alkalinization of the medium. Thus, the homeostasis of the external pH with the respiring cells represented a steady state in which the H^+ expelled through the respiratory pump was precisely equalled by the H^+ taken up by the cells, presumably for metabolic purposes. Several other agents caused a similar influx of H^+; the kinetics of this influx induced by some of the agents is given in Fig. 1. In all cases the H^+ influx appeared to reach a plateau level. The influx induced by CCCP was relatively slow; this was evidently because conductance to ions other than H^+ was rate limiting under these conditions, since the concurrent presence of valinomycin, which caused a marked K^+ efflux (2.7×10^{17} K^+ ions per mg of cell protein; as measured with a potassium electrode), greatly augmented the rate of CCCP-induced H^+ influx. The lipophilic anion thiocyanate, which of all the agents tested had the most pronounced effect on ΔpH in *T. acidophilus* (6), also caused a rapid H^+ influx and in accordance with previous observations (6) exhibited increased effectiveness at higher concentrations.

Table 1 presents the amounts of H^+ that were taken into the cells in response to treatments with various agents; the values were determined from the total change in the external pH observed when the H^+ influx approached a plateau (Fig. 1). The buffering capacity of the intact cells under these conditions was determined and found to be small; thus, it would not be considered in the results. Since the internal cell water volume was known (6), we calculated the expected cytoplasmic pH that this influx would produce in an unbuffered environment (Table 1). The actual cytoplasmic pH in the cells treated with the various agents for corresponding periods was measured by the transmembrane distribution of aspirin, using the flow dialysis technique (6), and is also presented in Table 1. It is evident that the cytoplasm possessed a strong buffering capacity since in all cases the actual cytoplasmic pH was 3 to 4 U higher than that expected from the H^+ influx. Repeated measurements, 20 in all, of CCCP-induced H^+ influx and the cytoplasmic pH in CCCP-treated cells (by flow dialysis) indicated that a buffering capacity of about 100 nmol of H^+ per mg of protein per pH unit would be required to account for this effect at pH 6.

To check this, the cytoplasmic buffering capacity was directly measured using disrupted cells, as described above. The extracts were titrated from pH 6 to 5, and an average value of 97 ± 41 nmol of H^+ per mg of protein per pH unit was obtained for the buffering capacity in four determinations. The buffering capacity in 5 and 10% Triton extracts was found to be very similar, suggesting that either concentration made the entire buffering capacity accessible. A very similar value for the buffering capacity was obtained using the acid pulse method described by Maloney (4) and Scholes and Mitchell (9). Thus, the measured buffering capacity of the cell extracts is equal to that predicted by the H^+ influx measurements described above and indicates that this capacity is indeed sufficient to account for the ability of the cells to maintain a relatively high cytoplasmic pH despite a large H^+ influx. The buffering capacity of *T. acidophilus* increased with a decreasing pH; from pH 5 to 4, this capacity, as measured in Triton extracts, was 260 nmol of H^+ per mg of protein per pH unit. For comparison, the buffering capacity of *Escherichia coli* (Triton extract) was determined from pH 6 to 5 and found to be 33 nmol of H^+ per mg of protein per pH unit.

The data presented clearly demonstrate that in actively respiring cells of *T. acidophilus*, H^+ ions flowing into the cells down their concentration gradient (presumably for metabolic purposes) are immediately expelled from the cells through the respiratory pump. Inhibition of this pump by anaerobiosis or treatment with the inhibitors of the respiratory chain prevents the H^+ extrusion but leaves the H^+ influx pathways intact for a considerable length of time. This results in a large net influx of H^+ into the cells. However, this influx (or that caused by treatment of cells with protonophores) has only a minimal effect on the cytoplasmic pH because of the buffering capacity of the cytoplasm, which is ca. 100 nmol of H^+ per mg of protein per pH unit; measurements established that this capacity is sufficient to account for the relative homeostasis of the cytoplasmic pH under metabolically inactive conditions.

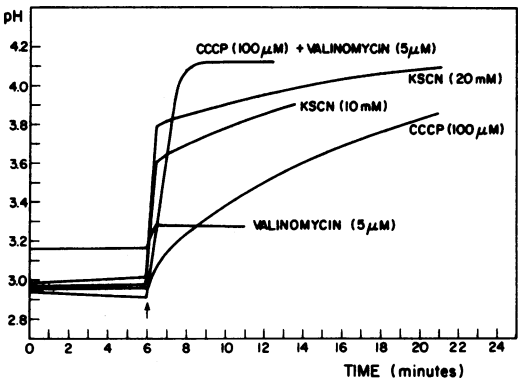

FIG. 1. Change in the external pH caused by the addition of different agents to a cell suspension of *T. acidophilus*.

It seems probable that the increase in the positive $\Delta\Psi$ that is observed under these conditions does indeed arise from the H^+ influx, as we proposed earlier (6). In all cases examined in this study, this influx was more than sufficient to account for the observed increase in $\Delta\Psi$. Thus, for instance, the CCCP-induced influx of H^+ was sufficient to cause an increase in $\Delta\Psi$ by some 120 mV, whereas the actual increase observed was some 30 mV (6); this discrepancy probably occurred because anion uptake or cation efflux accompanied the diffusion of H^+ into the cells. Regardless, once the positive $\Delta\Psi$ approaches the magnitude of the opposing ΔpH, a near-equilibrium situation is established in which no further significant H^+ influx into the cells occurs. The fact that a $\Delta\Psi$ of the order of +100 mV can be maintained for prolonged periods in metabolically inactive cells (10) indicates that the cytoplasmic membrane of these bacteria is unusually impermeable to ions other than H^+, as we suggested earlier (6).

It is not known what molecule(s) is responsible for buffering the H^+ activity. In other organisms (8), amino acid side chains of proteins have been implicated in this phenomenon, based on the finding that the cytoplasmic buffering power increased with a decreasing pH. The same is true for the buffering power of *T. acidophilus*, so that the cytoplasmic proteins may be the major buffers in this bacterium also, although it cannot be ruled out that the acidophiles possess some special molecule(s) for this purpose. Calculations have recently been made of the concentration of the nonpermeable monoprotonated molecule that would be needed to balance various magnitudes of ΔpH (1). These show that to maintain a ΔpH of ~2 U (i.e., the magnitude maintained for prolonged periods by nonmetabolizing cells of *T. acidophilus* [10]) a concentration of ca. 0.4 g-ion equivalent per liter would be required. As Cobley notes (1), this concentration is well within the physiological range. However, higher values of ΔpH observed in acidophiles under certain conditions cannot be explained on the basis of $\Delta\Psi$ generated by this mechanism. For instance, at an external pH of 1, *T. acidophilus* exhibits a ΔpH of 4.5 U (6), which would necessitate a concentration of the charged molecule of 3.2×10^3 g-ion equivalents per liter (1). This is clearly unattainable, and the ΔpH under these conditions must arise from other mechanisms, for instance, from greatly increased impermeability of the cell membrane to H^+ influx; indeed, we have preliminary evidence that little H^+ influx occurs in *T. acidophilus* cells at pH values of 2 and lower.

Although at optimal external pH values homeostasis of the cytoplasmic pH can be maintained by nonchemiosmotic means, active ionic pumping is nevertheless necessary for the survival of the organism. Data presented here and elsewhere (6, 10) show that the inhibition of respiration, with the resultant cessation of H^+

TABLE 1. Net H^+ influx and cytoplasmic pH in *T. acidophilus*

Agent inducing influx	Amt of H^+ influx[a]	Cytoplasmic pH	
		Estimated from H^+ influx[b]	Measured by flow dialysis[c]
Control	0		6.0
CCCP (100 μM)	1.1×10^{17}	1.0	5.2
Picric acid (50 μM)	1.7×10^{16}	1.8	5.3
Valinomycin (2 μM)	2.4×10^{16}	1.7	5.3
Nigericin (2 μM)	6.3×10^{15}	2.3	5.2
CCCP (100 μM) + valinomycin (5 μM)	2.3×10^{17}	0.7	4.8
Nigericin (2 μM) + valinomycin (5 μM)			5.2
NaSCN (10 mM)	1.1×10^{17}	1.0	5.0
NaSCN (20 mM)	1.3×10^{17}	0.9	4.5
NaSCN (20 mM) + CCCP (100 μM)	9.5×10^{16}	1.1	4.5

[a] H^+ ions per milligram of cell protein.

[b] Estimated from the total change in the external pH and the internal cell water volume. For instance, the addition of CCCP to cells caused a net change in the external pH from 3.015 to 3.951, i.e., a net H^+ uptake of 8.48×10^{-4} mol/liter. The reaction mixture contained 38 mg of cell protein in a total volume of 8 ml. Thus, 6.8×10^{-6} mol of H^+ was taken up by 75.8 μl of cell water, generating an internal H^+ concentration of 8.9×10^{-2} mol of water H^+ per liter, or a pH of 1.0.

[c] See reference 6 for the method used.

efflux, leads to a large influx of H^+, a virtual collapse of Δp, and a loss of culture viability.

Given the vicissitudes of nature, which ensure periods of starvation for virtually all bacteria, and given the crucial importance of maintaining cytoplasmic pH homeostasis, it is hardly surprising that *T. acidophilus* has evolved a passive capacity for achieving it. To what extent the various antiporters that are known to regulate the cytoplasmic pH in neutrophiles and alkalophiles (7) play a role in this bacterium remains to be determined.

This research was supported by grant PCM 8021571 from the National Science Foundation. E.Z. was supported by a predoctoral fellowship from Conacyt, Mexico.

We are grateful to Arturo Zychlinsky for his help in the preliminary part of this work.

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