The Protonmotive Force and the Δ pH in Spheroplasts of an Acidophilic Bacterium (*Thiobacillus acidophilus*)

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At an external pH of 3.0, spheroplasts of the acidophilic bacterium *Thiobacillus acidophilus* possessed a Δ pH of 2.9 units, a membrane potential of +86 mV, and a protonmotive force of –88 mV. The ultrastructure of the whole cells or spheroplasts showed no unusual features. At given concentrations, SCN^- was more effective in collapsing Δ pH in spheroplasts than in whole cells, indicating a lower permeability barrier in the former. However, the Δ p values in spheroplasts were very similar to those in the whole cells, and respiratory chain inhibitors, a protonophore, and an ATPase inhibitor, alone or in various combinations, were equally ineffective in collapsing Δ pH in whole cells and in spheroplasts. Thus, it is unlikely that the permeability barrier of the outer membrane accounted for the low values of measured Δ p, or the ineffectiveness of various agents in collapsing Δ pH in whole cells of this organism; this confirms our previous suggestion that a sizeable Δ pH in *T. acidophilus* can be maintained by non-chemiosmotic means.

INTRODUCTION

We have reported previously (Matin *et al.*, 1982) that at their optimal growth pH (3.0), *Thiobacillus acidophilus* cells maintained a Δ pH of 2.6–3.2 pH units, which was not affected significantly by protonophores, inhibitors of the respiratory chain, or *N,N'*-dicyclohexylcarbodiimide (DCCD), added individually or in various combinations. This would suggest that Δ pH in these bacteria can be maintained by non-chemiosmotic mechanisms. In contrast, in another acidophilic bacterium (*Bacillus acidocaldarius*), dinitrophenol completely abolished the Δ pH, and protonophores as well as respiratory chain inhibitors stopped protonmotive force (Δ p)-linked transport functions, leading to the conclusion that the maintenance of Δ pH in this bacterium was dependent on chemiosmotic mechanisms (Krulwich *et al.*, 1978). This discrepancy could have arisen from the fact that *T. acidophilus*, being Gram-negative, had a greater permeability barrier than *B. acidocaldarius*, which is Gram-positive; and indeed some of the experimental results did indicate a significant permeability barrier in *T. acidophilus*. Thus, SCN^- , which did collapse Δ pH in *T. acidophilus*, exhibited a concentration-dependent effect even in the high concentration range of 10–100 mM, and many agents showed a time-dependent effect. A permeability barrier to substrates like SCN^- , which was used as a probe for $\Delta\psi$ measurements, by preventing full equilibration between Δ p and the probes, might also have resulted in erroneous values of the measured Δ p.

In search of an experimental system that would circumvent the permeability barrier problem, we attempted to make right-side-out membrane vesicles of *T. acidophilus*, using the method described by Kaback (1967). These attempts were not successful, but it proved possible to make spheroplasts of this bacterium that retained Δ pH and $\Delta\psi$, and exhibited a markedly diminished permeability barrier compared with whole cells. Consequently, we have examined the effect of a number of agents on Δ p in spheroplasts of *T. acidophilus*.

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide.

METHODS

The organism and culture conditions were exactly as described previously (Matin *et al.*, 1982). The procedure for flow-dialysis was similar to that previously employed with whole cells, except that the reaction mixtures and the effluent buffer contained 20% (w/v) sucrose to stabilize the spheroplasts. Respiration was measured as described previously (Matin & Konings, 1973).

Spheroplasts were prepared as follows. Cells were harvested by centrifugation (9000 *g*, 20 min, 4 °C), washed once in 30 mM-Tris/HCl buffer (pH 8), and suspended in the same buffer to a density of 5 mg cell protein ml⁻¹. EDTA, sucrose, and lysozyme were added to concentrations of 10 mM, 20% and 1 mg ml⁻¹, respectively, and the suspension was incubated on a shaker at 37 °C for 16 h. The incubation period was determined in control experiments; incubation for < 16 h produced only partial conversion of the cell suspension to spheroplasts. The spheroplasts were washed in Tris/HCl buffer (supplemented with 20% sucrose), and suspended in β -alanine buffer (0.1 M; pH 3.0), containing 20% sucrose.

For electron microscopy, whole cells of *T. acidophilus* were fixed for 6.5 h at 4 °C in 1% (w/v) glutaraldehyde in 0.067 M-potassium phosphate buffer (pH 7.0) supplemented with 0.4% glucose. After washing in phosphate buffer/glucose solution, the cells were post-fixed overnight at 4 °C in 1% OsO₄ solution in phosphate buffer/glucose. Cells were then washed in 0.1 M-sodium acetate buffer (pH 6.0) and stained for 1–2 h with 1% (w/v) uranyl acetate in sodium acetate buffer. A similar procedure was used for the spheroplasts, except that 0.1 M-potassium phosphate buffer (pH 7.0) supplemented with 20% sucrose was used in place of potassium phosphate/glucose solution for the glutaraldehyde and OsO₄ fixations. OsO₄ fixation was followed by a water rinse and treatment with 1% aqueous uranyl acetate. Both the whole cell and the spheroplast preparations were dehydrated in increasing concentrations of ethanol, embedded in Epon, sectioned in a Sorval MT28, and photographed in a Philips 201C electron microscope at 80 kV.

RESULTS

There were no unusual features in the ultrastructure of whole cells of *T. acidophilus* (Fig. 1), and as in other Gram-negative bacteria, the cell envelope consisted of a thin layer of peptidoglycan sandwiched between the unit cytoplasmic and outer membranes. Similarly, the ultrastructure of spheroplasts resembled that of *Escherichia coli* (DePetris, 1967): the peptidoglycan was missing and the outer membrane appeared somewhat loose and stretched. The inclusions (Fig. 1) were probably poly- β -hydroxybutyric acid granules (Matin *et al.*, 1979), since chemical analyses revealed significant quantities of this polymer in the cells. The spheroplasts exhibited active respiration, which was completely abolished by 10 mM-azide, as was the case in whole cells (Matin *et al.*, 1982).

The Δ pH and $\Delta\psi$ values were measured in spheroplasts by flow-dialysis. The value of the Δ pH was 2.9 ± 0.27 units (average of 14 independent measurements $\pm$ S.E.), and that of $\Delta\psi$ was $+86 \pm 6.5$ mV (average of 5 independent measurements $\pm$ S.E.); thus, the value of Δp was -88 mV. These values are very similar to those found in whole cells of this bacterium (Matin *et al.*, 1982).

A comparison of the effect of exposure to SCN⁻ on Δ pH in the spheroplasts and the whole cells showed (Fig. 2) that 5 mM-SCN⁻ collapsed Δ pH to a greater extent in the former: the residual Δ pH in the spheroplasts was 1.8 (a decrease of 1.2 units), whereas in the whole cells it was 2.4 (a decrease of 0.5 unit). SCN⁻ is believed to collapse Δ pH by neutralizing the positive membrane potential (Krulwich *et al.*, 1978; Maloney, 1979; Matin *et al.*, 1982; Mitchell & Moyle, 1967), for which its entry into the cells (or spheroplasts) is necessary. It therefore follows that it can permeate the spheroplast more readily than the whole cells. The greater effectiveness of SCN⁻ in collapsing Δ pH in spheroplasts was also evident at several additional concentrations of the anion (Fig. 3).

Similar differences were found in the ability of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (100 μ M) plus SCN⁻ (5 mM) to collapse Δ pH in whole cells and spheroplasts. In the former case, 15 min incubation with these agents produced a 50% collapse in Δ pH, whereas in the latter case, these conditions caused a complete collapse in Δ pH.

Having established that the spheroplasts do indeed have a decreased permeability barrier compared with whole cells, we examined the effect of various agents on their Δ pH. None of the agents tested, which included a protonophore, an ATPase inhibitor, and respiratory chain

Fig. 1. Ultrastructure of whole cells and spheroplasts of *T. acidophilus*. (a) Longitudinal section of whole cell; (b) close-up of outer envelope in whole cell; (c) cross-section of whole cell; (d) longitudinal section of spheroplast.

Table 1. Effect of various agents on ΔpH in spheroplasts of *T. acidophilus*

Agent	ΔpH	Decrease in ΔpH (% of control)
None	3.0	—
CCCP (100 μM)	2.3	23
Azide (20 mM)	2.0	33
Antimycin A [5 μg (mg protein) ⁻¹]	2.7	10
DCCD (10 μM)	2.9	3
Azide + DCCD (20 mM and 10 μM , respectively)	1.9	37

inhibitors, tested individually or in various combinations, had any marked effect on ΔpH of the spheroplasts (Table 1): in all cases, >60% of the initial ΔpH was retained. This effect is very similar to that observed with whole cells (Matin *et al.*, 1982).

Fig. 2. Effect of KSCN on ΔpH in whole cells (a) and spheroplasts (b) of *T. acidophilus*. ●, Control experiments (KSCN not present); ▲, test experiments (5 mM KSCN in the reaction mixture). Calculated ΔpH values were: whole cell control, 2.9; spheroplast control, 3.0; whole cell test, 2.4; spheroplast test, 1.8. There was no further change in test ΔpH values in the whole cells or the spheroplasts when incubation with KSCN was prolonged to 15 min. The amount of protein in the reaction mixture was $1.6 \text{ mg } (0.8 \text{ ml})^{-1}$ for whole cells and $4 \text{ mg } (0.8 \text{ ml})^{-1}$ for spheroplasts.

Fig. 3. Effect of various concentrations of potassium thiocyanate on ΔpH in *T. acidophilus* spheroplasts (●), and whole cells (▲). The whole cell data are reproduced from Matin *et al.* (1982) for comparison.

DISCUSSION

We have shown here that the spheroplasts of *T. acidophilus* retain the capacity to maintain a ΔpH , and that they possess an impaired permeability barrier compared with whole cells, presumably because of a damaged outer membrane. Their increased permeability offered the possibility for a more quantitative assessment of Δp value in this organism, since it would conceivably allow for a fuller equilibration of Δp parameters with the probe molecules than might be possible in whole cells. However, the ΔpH , $\Delta\psi$, and Δp values measured in spheroplasts were found to be very similar to those determined using whole cells of this bacterium (Matin *et al.*, 1982), and it therefore appears unlikely that the outer-envelope permeability barrier of the whole cell had influenced the measured Δp value. We conclude that Δp in *T. acidophilus* does indeed lie in the vicinity of -80 mV . The question of how these bacteria drive their proton-consuming reactions at such a low value of Δp (Matin *et al.*, 1982) is thus brought into a sharper focus. Some of the obvious possibilities are that these reactions equilibrate with Δp extant in localized regions of the membrane (Guffanti *et al.*, 1981; Williams, 1978), or that the proton stoichiometries are unusually high in these bacteria.

The increased permeability of the spheroplasts made it possible to approach the question of whether the ineffectiveness of various protonophores and inhibitors in collapsing ΔpH in whole cells was due to the permeability impediment of the cells. Had that been the case, one would

expect the ΔpH in spheroplasts to be more readily collapsible by these agents. In fact, however, the spheroplasts exhibited no greater sensitivity than the whole cells, so that it is unlikely that the permeability barrier accounted for the resistance of ΔpH in *T. acidophilus* to the various agents. Our recent findings involving direct monitoring of ionic movements are consistent with this conclusion: they show that a number of protonophores and DCCD influence H^+ extrusion and uptake by whole cells of this bacterium in a manner consistent with their established mode of action (E. Zychlinsky & A. Matin, unpublished). We conclude that a sizeable ΔpH can in fact be maintained in *T. acidophilus* by non-chemiosmotic means.

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