Transmembrane Electrical Potential and Transmembrane pH Gradient in the Acidophile *Thiobacillus ferro-oxidans*

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Thiobacillus ferro-oxidans is capable of using the oxidation of Fe^{2+} by O_2 at pH 2.0 as the sole source of energy for growth and CO_2 fixation. The bacterium maintains an intracellular pH of 6.5 over a range of external pH from 1.0 to 8.0, as measured by [^{14}C]acetate and [^3H]methylamine distribution. The membrane potential was estimated by the distribution of the lipid-soluble cation dibenzyltrimethylammonium and the anion SCN^- . At pH 2.0 (the pH of growth) during Fe^{2+} oxidation the transmembrane pH gradient is 4.5 units with an opposing membrane potential of -10mV , giving a proton electrochemical gradient of $+256\text{mV}$. This gradient is actively maintained.

The problem of how growth of bacteria at extreme pH can be reconciled with a chemiosmotic mode of energy conservation, given the need to maintain an internal pH near neutrality, has been considered by Garland (1977). In the present paper we show that *Thiobacillus ferro-oxidans*, a bacterium that grows optimally at pH 2.0 with Fe^{2+} as sole energy source, maintains a transmembrane pH gradient of 4.5 units together with a small negative membrane potential. Although the relative contributions of ΔpH and $\Delta\psi$ to the proton electrochemical gradient in this organism are unique, the net result is entirely compatible with a chemiosmotic mechanism of energy conservation. Ingledew *et al.* (1977) proposed that the transmembrane pH gradient is maintained by the removal of H^+ by the reduction of oxygen to water in the terminal oxidase reaction in the cell matrix. They also proposed that ATP was synthesized conventionally by protons entering the cell through a proton-translocating ATPase driven by $\Delta\bar{\mu}_{\text{H}^+}$.

ΔpH has been measured previously in two other acidophilic bacteria, *Thermoplasma acidophila* (Hsung & Haug, 1975) and *Bacillus acidocaldarius* (Oshima *et al.*, 1977). Hsung & Haug (1975) grew *Thermoplasma acidophila* at pH 2 and by using the distribution of 5,5-dimethyl-2,4-oxazolidinedione

measured a ΔpH of +4 units (i.e. alkaline inside), which was not destroyed by boiling or by metabolic inhibitors. In contrast, Searcy (1976), also using *Thermoplasma acidophila*, obtained a ΔpH of +3.5 units, which was destroyed by boiling. In the other acidophilic bacterium, *Bacillus acidocaldarius*, Oshima *et al.* (1977) showed that the internal pH was maintained near neutrality when the external pH was 3.0, the pH gradient again being monitored by the distribution of 5,5-dimethyl-2,4-oxazolidinedione. In the only previous attempt to measure the full $\Delta\bar{\mu}_{\text{H}^+}$ of an acidophilic bacterium, Hsung & Haug (1977) obtained a value of approx. 145mV , composed of a ΔpH of +4.5 units and a $\Delta\psi$ of -120mV (measured by SCN^- distribution). The membrane potential was unaffected by boiling or by millimolar concentrations of 2,4-dinitrophenol or carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

The purpose of the present paper is to report values for ΔpH , $\Delta\psi$ and $\Delta\bar{\mu}_{\text{H}^+}$ in *Th. ferro-oxidans* over an external pH range of 1.0–8.0. We conclude that a chemiosmotic mechanism of energy conservation is entirely compatible with an acidophilic existence. The only constraint being imposed by growth in acidic environments is that the proton electrochemical gradient is largely in the form of a pH gradient.

Materials and Methods

Growth and harvesting

Th. ferro-oxidans (strain T.f.3), obtained from the Microbiological Research Establishment, Porton Down, Salisbury, Wilts., U.K., was grown and

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Abbreviations: ΔpH , transmembrane pH gradient (pH outside–pH inside); $\Delta\psi$, membrane potential (potential outside–potential inside); $\Delta\bar{\mu}_{\text{H}^+}$, proton electrochemical gradient.

harvested as described by Cobley & Haddock (1975). Some 3–5 g wet wt. of *Th. ferro-oxidans* was washed twice in 10 mM-H₂SO₄, pH 2.0, and twice in water to remove Fe²⁺ and precipitated Fe(OH)₃. Cells were resuspended in water to a protein concentration of approx. 20 mg/ml.

Measurement of ΔpH and $\Delta\psi$

Gradients were determined by isotope distribution (Nicholls, 1974). ΔpH was measured by the distribution of [³H]acetate or [¹⁴C]methylamine, and $\Delta\psi$ by the distribution of S¹⁴CN[−] or [³H]dibenzyltrimethylammonium. ³H₂O and [¹⁴C]sucrose were used as reference radioisotopes. The measuring ions used, including reference radioisotopes, were (per ml of incubation): $\Delta\psi > 0$, 0.075 μCi of [³H]dibenzyltrimethylammonium (20 μM) + 0.06 μCi of [¹⁴C]sucrose; $\Delta\psi < 0$, 0.025 μCi of S¹⁴CN[−] (1 μM) + 0.3 μCi of ³H₂O; $\Delta\text{pH} < 0$, 0.03 μCi of [³H]acetate (20 μM) + 0.06 μCi of [¹⁴C]sucrose; $\Delta\text{pH} > 0$, 0.075 μCi of [¹⁴C]methylamine (25 μM) + 0.3 μCi of ³H₂O. All incubations were carried out in triplicate at 22°C in a total volume of 800 μl , comprising the appropriate radioactive mediators in 790 μl of 5 mM-buffer (pH range 1.0–8.0) and 2 mM-FeSO₄ or ascorbate. Cells were added to a final concentration of approx. 0.5 mg of protein/ml. Samples (200 μl) were filtered through Sartorius cellulose nitrate filters (pore size 0.6 μm) 30, 60 and 90 s after addition of the cells. Calculations were performed on cells filtered after 60 s, but no significant differences were observed if cells filtered after 30 or 90 s were used. The filters were transferred directly, without washing, to scintillation vials containing 5 ml of scintillation fluid (4 g of 2,5-diphenyloxazole, 310 ml of Triton X-100, 630 ml of toluene and 60 ml of water). The radioactivity was counted in an InterTechnique SL 30 liquid-scintillation counter. The buffers used were: for pH 1.0, 50 mM-H₂SO₄; for pH 2.0, 10 mM-H₂SO₄; for pH 3.0, 10 mM- β -alanine sulphate; for pH 4.0, 10 mM- β -alanine sulphate; for pH 5.0, 10 mM-4-morpholine-ethanesulphonic acid; for pH 6.0, 10 mM-4-morpholine-ethanesulphonic acid; for pH 7.0, 10 mM-potassium phosphate; for pH 8.0, 10 mM-Tris/HCl.

The matrix volume was determined from the difference between the [¹⁴C]inulin- or sucrose-permeable space and the ³H₂O-permeable space (Rottenberg *et al.* 1972). When either sucrose or inulin was used as the impermeable solute the matrix volume was found to be 2.4 ± 0.1 (4) $\mu\text{l}/\text{mg}$ of protein.

Calculation of results

ΔpH (< 0) was calculated from the distribution of [³H]acetate by the equation of Addanki *et al.* (1967, 1968):

$$\text{pH}_{\text{in}} = \text{pK}_a + \log_{10} \left[\frac{[\text{Ac}]_{\text{in}}}{[\text{Ac}]_{\text{out}}} (1 + 10^{\text{pH}_{\text{out}} - \text{pK}_a}) - 1 \right] \quad (1)$$

ΔpH (> 0) and $\Delta\psi$ were calculated as previously described by Nicholls (1974).

Other techniques

Rates of oxygen consumption were assayed in a Clark oxygen electrode. Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Reagents

Radioisotopes were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. (the [³H]dibenzyltrimethylammonium being synthesized by special order). Bovine serum albumin was obtained from Sigma Chemical Co., Poole, Dorset, U.K. All other chemicals were of the AnalaR grade and supplied by BDH Chemicals, Poole, Dorset, U.K.

Results and Discussion

Measurement of intracellular pH at various external pH values

Th. ferro-oxidans cells can oxidize Fe²⁺ between pH 1.0 and 4.0 and ascorbate between pH 1.0 and 8.0. Ingledew *et al.* (1977) have used the distribution of azide (the ion of a weak monobasic acid) to locate the site of oxygen reduction by the cytochrome oxidase of *Th. ferro-oxidans*. They also obtained a value of 5.5–6.0 for the intracellular pH based on the assumption that azide will distribute across the cell membrane in accordance with the transmembrane pH gradient (Palmieri & Klingenberg, 1967).

The use of a method that measures the internal pH by virtue of inhibition of ascorbate oxidation may lead to a disturbance of the pH gradient in the metabolically poisoned cells. So it was necessary to use a confirmatory method which did not depend on, or cause, inhibition of Fe²⁺ or ascorbate oxidation.

The results obtained from the distribution of [³H]acetate and [¹⁴C]methylamine in respiring and heat-denatured cells are shown in Fig. 1. Fig. 1(a) shows the relation between the matrix pH and the pH of the bulk phase during ascorbate oxidation. Similar results were obtained between pH 1.0 and 3.0 when Fe²⁺ was the substrate. In Fig. 1(b) the data are replotted to show the variation of the ΔpH with changing external pH. In heat-denatured cells no ΔpH can be detected. In respiring cells the internal pH remained close to pH 6.5, varying by less than 0.5 pH unit over the whole external pH range. At pH 2.0 the pH gradient was -4.5 units, corresponding to a $-59\Delta\text{pH}$ of 266 mV.

Fig. 1. Variation of internal pH and pH gradient over the external pH range 1.0–8.0

Washed cells (0.4 mg of protein/ml of incubation mixture) were incubated at 22°C in media buffered over the pH range 1.0–8.0 and containing 2.5 mM-ascorbate and the following radioisotopes to indicate the transmembrane pH gradient: [³H]acetate plus [¹⁴C]sucrose (○) or [¹⁴C]methylamine plus ³H₂O (Δ). Controls were performed as above but with cells that had been boiled for 1 h. (□). (a) Magnitude of matrix pH as the external pH is varied; (b) magnitude of transmembrane pH gradient as the external pH is varied.

Fig. 2. Effect of SCN⁻ on oxygen uptake by *Th. ferro-oxidans*

Experiments were performed in a Clark oxygen electrode, thermostatically controlled at 30°C. Working volume was 4 ml containing 0.25 mg of cells/ml and 4 ml of 10 mM-H₂SO₄ buffer, pH 2.0. (a) +2 μM-SCN⁻; (b) +2 μM-SCN⁻ and 5 μM-2,4-dinitrophenol. Oxygen uptake was started by addition of 2 μM-Fe²⁺ to the incubation mixture, and SCN⁻ was added at the points indicated by arrows.

The internal pH determined by isotope distribution is slightly higher than that estimated by Ingledew *et al.* (1977) using azide distribution. The reason for this discrepancy is probably that azide is inhibiting

the proton-consuming oxidase reaction, and hence the internal pH will be slightly more acid. Acetate inhibits ascorbate and Fe²⁺ oxidation at high concentration (50% inhibition requiring 30 mM-acetate at pH 2.0), but has no inhibitory effect at the concentrations used here (20 μM).

Use of SCN⁻ in determination of membrane potential

It is established that SCN⁻ will distribute according to the $\Delta\psi$ at neutral pH (Rottenberg, 1975). However, we use this anion to monitor $\Delta\psi$ at pH 1.0, and it is essential to demonstrate the validity of the technique at acidic pH.

The pK_a of thiocyanic acid (HSCN) is -1.85 (Morgan *et al.*, 1965), therefore at pH 1.0 there will be 0.14% of the undissociated acid present (i.e. 1.4 nM under experimental conditions). Although this is a significant fraction, the following evidence suggests that it is the SCN⁻ anion that is accumulated, uptake being driven almost entirely by electrogenic entry. We have found that, over the pH range 1.0–3.0, SCN⁻ inhibits oxygen uptake by *Th. ferro-oxidans* by a mechanism that remains to be established. Fig. 2(a) shows the effect of 2 μM-SCN⁻ on Fe²⁺ oxidation by *Th. ferro-oxidans* at pH 2.0, the initial rate of oxygen uptake being inhibited by about 20%. Fig. 2(b) depicts a similar experiment, except that 5 μM-2,4-dinitrophenol is present. It is clear that, in the presence of uncoupler, SCN⁻ is a much more

Table 1. *Effect of respiratory inhibitors and uncouplers on $\Delta\bar{\mu}_{H^+}$*

Cells were incubated at pH 2.0 as described in the Materials and Methods section. $S^{14}CN^-$ was used to determine $\Delta\psi$, and was present at $1\ \mu M$ in experiment (a) and $27\ \mu M$ in experiments (b) and (c). $[^3H]$ Acetate ($20\ \mu M$) was used to determine ΔpH .

Experiment	2,4-Dinitrophenol (μM)	Azide (mM)	$\Delta\psi$ (mV)	$-59\Delta pH$ (mV)	$\Delta\bar{\mu}_{H^+}$ (mV)	pH _{in}
(a)	0	0	-10	+266	+256	6.5
(b)	0	0	-134	+277	+143	6.7
	8.3	0	-188	+244	+56	6.1
	25.0	0	-192	+242	+50	6.1
(c)	0	1.0	-184	+242	+58	6.1

Fig. 3. *pH-dependence of SCN^- inhibition of Fe^{2+} oxidation in whole and broken cells of *Th. ferro-oxidans**

Experiments were performed in a Clark oxygen electrode at $30^\circ C$. Incubation conditions were as described in the legend to Fig. 2. Cells were broken by preincubation for 10 min with 2.5% Triton X-100, or by sonication. The buffers were: pH 1.0, 50 mM- H_2SO_4 ; pH 1.5, 30 mM- H_2SO_4 ; pH 2.0, 10 mM- H_2SO_4 ; pH 3.0, 10 mM- β -alanine sulphate. $\circ$, Intact cells; Δ , broken cells.

efficacious inhibitor of Fe^{2+} oxidation in intact cells. The uncoupler permits entry of protons into the cell, which will cause an increase in the membrane potential (negative outside) and a decrease in the pH gradient (Table 1). It is likely therefore that SCN^- distributes in accordance with the $\Delta\psi$, as it is a more effective inhibitor of Fe^{2+} oxidation under conditions where the $\Delta\psi$ is increased. Similar results were obtained with ascorbate as the oxidizable substrate, indicating that an iron- SCN^- complex was not responsible for the inhibition.

Fig. 3 also indicates that SCN^- responds to the $\Delta\psi$. Inhibition of Fe^{2+} oxidation in intact and broken cells requires the same amount of SCN^- at pH 2.0, suggesting an accumulation ratio of unity for the

cells at this pH. This is markedly different from the point of zero pH gradient as determined by azide or $[^3H]$ acetate distribution (pH 5.5–6.5). By analogy with the azide-uptake experiments of Ingledew *et al.* (1977), the difference in effectiveness of SCN^- inhibition between intact and broken cells suggests that the inhibitory action of SCN^- is due to its uptake.

The polarographic experiments further served to enable the determination of a concentration of SCN^- which would not cause underestimation of the $\Delta\psi$ due to inhibition of Fe^{2+} or ascorbate oxidation by *Th. ferro-oxidans*; 50% inhibition occurs at $1.5\ \mu M$ - SCN^- at pH 1.0 (Fig. 3). In experiments using $S^{14}CN^-$ distribution a final concentration of $1.0\ \mu M$ - SCN^- was used, and this did not greatly inhibit oxygen uptake and is unlikely to cause underestimation of the magnitude of the $\Delta\psi$.

Fig. 3 also allows a qualitative estimation of $\Delta\psi$ to be made by comparing the concentration of SCN^- required to cause 50% inhibition of Fe^{2+} oxidation in the intact and broken cells. SCN^- is a more effective inhibitor of intact rather than broken cells below pH 2.0, the anion apparently being accumulated. Above pH 2.0 the SCN^- is apparently excluded, indicating that at pH 2.0 the sign of the membrane potential reverses.

Experiments with $S^{14}CN^-$ to monitor $\Delta\psi$ showed that at pH 1.0 respiring cells had a $\Delta\psi$ of $-70\ mV$, rising to $-10\ mV$ at pH 2.0 and to zero at pH 3.0. These values fell to $-95\ mV$ at pH 1.0, $-65\ mV$ at pH 2.0 and $-25\ mV$ at pH 3.0 in the presence of $5\ \mu M$ -2,4-dinitrophenol.

Measurement of $\Delta\psi$ by using the cation dibenzyl-dimethylammonium

The use of dibenzyl-dimethylammonium to determine $\Delta\psi$ has been reviewed by Skulachev (1971). The experiments described in this section make use of the anion tetraphenylboron to facilitate entry of the dibenzyl-dimethylammonium cation (Skulachev, 1971). Fig. 4 shows the results obtained with $[^3H]$ dibenzyl-dimethylammonium in the presence of $1\ \mu M$ -tetraphenylboron over the pH range 2.0–8.0.

Fig. 4. $\Delta\psi$ at various external pH values. Incubation conditions were as described in the Materials and Methods section. The following radioisotopes were used to determine $\Delta\psi$: S¹⁴CN⁻ (○); [³H]dibenzyltrimethylammonium plus 1 μ M-tetra-phenylboron (Δ). S¹⁴CN⁻ is unable to detect a $\Delta\psi$ (>0), i.e. at pH values greater than 2.0.

Accumulation occurs at pH 3.0 and above, indicating that a $\Delta\psi$ (>0) is developed. This is in agreement with the data of Fig. 3, which suggest that the $\Delta\psi$ is positive under these conditions. The $\Delta\psi$ reaches a maximum value of +170 mV at pH 8.0 during ascorbate oxidation. Boiling the cell suspension causes a collapse of the $\Delta\psi$ (results not shown).

Magnitude of the proton electrochemical gradient

Fig. 5 shows the magnitude of the $\Delta\bar{\mu}_{H^+}$ over the pH range 1.0–8.0 for ascorbate oxidation and 1.0–3.0 for Fe²⁺ oxidation. At pH 2.0, the optimum pH for growth, the $\Delta\bar{\mu}_{H^+}$ of 256 mV is composed entirely of the pH gradient. This value is among the highest reported for bacteria (Rottenberg, 1975). Boiling the cells resulted in the total collapse of the $\Delta\bar{\mu}_{H^+}$, both the $\Delta\psi$ and the Δ pH coming to zero.

Fig. 5. Magnitude of the proton electrochemical gradient at various external pH values during the oxidation of Fe²⁺ or ascorbate by *Th. ferro-oxidans*.

This was calculated from the data of Figs. 1 and 5 by using the equation $\Delta\bar{\mu}_{H^+} = \Delta\psi - 59\Delta$ pH. ○, Ascorbate oxidation; Δ , boiled cells; □, Fe²⁺ oxidation.

Effect of respiratory inhibitors and uncouplers on the $\Delta\bar{\mu}_{H^+}$

As azide and high concentrations of SCN⁻ inhibit respiration, it could be predicted that they should lower the $\Delta\bar{\mu}_{H^+}$. Table 1 shows that increasing the concentration of SCN⁻ from 1 to 27 μ M lowers the $\Delta\bar{\mu}_{H^+}$ from 256 to 143 mV, entirely as a result of decreasing the $\Delta\psi$ from -10 mV to -134 mV. This concentration of SCN⁻ is twice that required for 50% inhibition of respiration at pH 2.0 (Fig. 3). The further addition of 1 mM-azide, which completely inhibits respiration at pH 2.0 (Ingledew *et al.*, 1977), lowers the $\Delta\bar{\mu}_{H^+}$ to 58 mV, again mainly by decreasing $\Delta\psi$. As would be predicted, 2,4-dinitrophenol lowers $\Delta\bar{\mu}_{H^+}$ to 50 mV. It is significant that the internal pH remains above 6.0, even when $\Delta\bar{\mu}_{H^+}$ is lowered by 200 mV. Thus, whereas heat denaturation abolishes both components of $\Delta\bar{\mu}_{H^+}$, respiratory inhibitors and uncouplers lower $\Delta\bar{\mu}_{H^+}$ while maintaining the Δ pH. This is in conflict with the results of Hsung & Haug (1975), who found that boiling cell suspensions did not apparently abolish Δ pH in *Thermoplasma acidophila*, and also contrasts with the results of Searcy (1976) and Oshima *et al.* (1977), who found that both boiling and metabolic inhibitors did cause the collapse of Δ pH in acidophiles.

One possible reason for this discrepancy is that Hsung & Haug (1975), Searcy (1976) and Oshima *et al.* (1977) used 5,5-dimethyl-2,4-oxazolidinedione

as an indicator of the pH gradient. This has a pK_a of 6.5 and if the uncharged form is permeable it will equilibrate equally on both sides of the membrane. The internal pH of *Thermoplasma acidophila* is proposed by Hsung & Haug (1975) to be 6.5. At an external pH of 2.0 (where a 1 mM solution of 5,5-dimethyl-2,4-oxazolidinedione would be virtually 100% protonated) the 5,5-dimethyl-2,4-oxazolidinedione would only enter the cell until there was an internal concentration of the protonated form equal to 1 mM. As the pK_a of 5,5-dimethyl-2,4-oxazolidinedione is 6.5 this would occur when the concentration gradient of radioisotope was only 2-fold. As a difference between a pH gradient of 4.5 units and one of zero under these conditions is only reflected in a 2-fold change in accumulation, the use of this indicator is inappropriate. In the present work, acetic acid (pK_a 4.75) or azide (pK_a 4.3) were used as indicators of the pH gradient. With acetate, at an internal pH of 6.5, it would be possible to obtain an accumulation ratio of acetate anion over the uncharged form of 100-fold.

Conclusion

Th. ferro-oxidans can maintain the pH of the matrix in the range 6.0–7.0 under all the conditions examined in this paper (with the exception of heat denaturation). The use of acetate as an indicator of ΔpH has enabled the determination of the matrix pH with far greater precision than is possible with 5,5-dimethyl-2,4-oxazolidinedione; nevertheless, the ability of the cells to maintain their internal pH, even when $\Delta\bar{\mu}_{H^+}$ is virtually abolished, tends to support

the suggestion of Hsung & Haug (1975) that the maintenance of the internal pH is not an energy-dependent process.

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