Dissipation of the Proton Motive Force in Oral Streptococci by Fluoride

SHELBY KASHKET\(^*\) AND EVA R. KASHKET\(^2\)

Forsyth Dental Center, Boston, Massachusetts 02115,\(^1\) and Department of Microbiology, Boston University School of Medicine, Boston, Massachusetts 02118\(^2\)

Received 22 October 1984/Accepted 8 January 1985

Strains of oral streptococci maintain an intracellular pH (pH\(_i\)) that is more alkaline than the external pH (pH\(_e\)). The ΔpH (pH\(_e\)−pH\(_i\)) was about 0.6 in neutral media and about 0.9 in media of pH 5.5. Addition of 10 μg of F\(^−\) per ml at pH 7.0 reduced the ΔpH in Streptococcus salivarius to 0.17. The reduction of ΔpH in S. sanguis H7PR3 was less pronounced, whereas the ΔpH in S. mutans IB 1600 was unaffected. The F\(^−\)-resistant mutant of S. salivarius, strain Flr103, maintained a ΔpH of 0.51 with 100 μg of F\(^−\) per ml. Addition of F\(^−\) to cells in media below pH 6.0 led to a reduction of ΔpH in all strains. The anion had no effect on the transmembrane electrical gradient of either mutant or parental cells of S. salivarius at pH 7.0. The principal effect of F\(^−\) addition at neutral pH, therefore, was on the ΔpH component of the proton motive force. At pH 5.5, 100 μg of F\(^−\) per ml reduced the transmembrane electrical gradient from 71 to 40 mV in the parent and from 80 to 42 mV in the mutant. We propose that the greater sensitivity of cells to F\(^−\) at lower medium pH stems from the rapid dissipation of ΔpH by the anion. Thus, pH equilibration in media of low pH would lead to a greater reduction of metabolic activity than when it occurs in media at neutral pH. The data also suggest that the growth of streptococci, with or without added F\(^−\), is limited when the intracellular pH falls below about 5.7.

Fluoride is being used extensively as an anticaries agent, although the mechanism by which the protective effect is brought about is not fully understood. In recent years, attention has shifted from an almost exclusive focus on fluoride-enamel interactions to the antimetabolic action of the anion. Impetus was given to these studies by the finding that plaque contains F\(^−\) and that plaque organisms can accumulate added F\(^−\) (3, 4, 10, 12, 14, 20, 28, 29). A number of investigators have shown that F\(^−\) taken up by oral microorganisms has an inhibitory effect on glycolysis (3, 6, 7, 15, 23, 26), and Bunick and Kashket (2) showed that streptococcal enolase is inhibited by F\(^−\). Presumably, the resulting drop in phosphoenolpyruvate production leads to a reduction in phosphotransferase-mediated sugar transport into the cells.

Metabolic energy is conserved in bacteria by an electrochemical proton gradient across the cytoplasmic membrane (8). This gradient, which consists of the transmembrane chemical gradient of H\(^+\) ions (ΔpH) and a transmembrane electrical gradient (Δφ), gives rise to the proton motive force (Δp) (21, 22). The Δp is established and maintained by the H\(^+\)-ATPase which utilizes glycolytically generated ATP. In metabolically active streptococci, the interior of the cell is alkaline and negative compared with the external environment (9, 11).

Since F\(^−\) inhibits glycolysis, we undertook to examine the effects of F\(^−\) addition on the components of Δp. Experiments were carried out with several strains of oral streptococci with different sensitivities to F\(^−\) (15) to determine whether strain-specific differences exist in the response of the components of Δp to F\(^−\). Portions of these studies have been presented in preliminary form (13, 25).

MATERIALS AND METHODS

Growth of streptococci. Streptococcus salivarius ATCC 25975, the fluoride-resistant mutant Flr103 derived from it by selection with 5.3 mM NaF (100 μg of F per ml) (2), and S. sanguis H7PR3 were grown at 37°C in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.). S. mutans IB 1600 was grown in brain heart infusion (Difco Laboratories, Detroit, Mich.). S. sanguis H7PR3 and S. mutans IB 1600 were obtained from the culture collection of the Forsyth Dental Center.

Measurement of pH. The cells were grown to late-exponential phase, centrifuged, and suspended in 0.15 M NaCl to give approximately 5 mg (wet weight) of cells per ml. Intracellular pH (pH\(_i\)) was measured by the distribution of 7-[\(^14\)C]benzoic acid across the bacterial cell membranes (19). The extracellular aqueous volumes of the pelleted cells were determined from the exclusion of 1.2-[\(^14\)C]polyethylene glycol (14). The Δφ values were measured by the uptake of [\(^3\)H]tetraphenylphosphonium (11). In a typical experiment, 0.20 ml of the stock cell suspension was added to a mixture containing 1.0 ml of buffer (either 100 mM sodium acetate, 75 mM KH\(_2\)PO\(_4\), 1 mM MgSO\(_4\), 10 μM EDTA [pH 5.5] or 75 mM Na\(_2\)HPO\(_4\), 75 mM KH\(_2\)PO\(_4\), 1 mM MgSO\(_4\), 10 μM EDTA [pH 7.0]) supplemented with 0.20 ml of 100 mM glucose containing 10 mM MgSO\(_4\), 0 to 0.20 ml of 5.3 mM NaF as indicated, the radioactive probes, and water to a total volume of 2.0 ml. The mixtures were incubated for 15 min at 23°C, and the extracellular pH (pH\(_e\)) was measured. Samples (1 ml) of the mixture were removed, and the cells were separated by centrifugation through silicone oil (11). The oil and aqueous phases were discarded, and the pellets were removed by cutting the tips off the microfuge tubes. Radioactivity in the pellets was determined in a Beckman liquid scintillation system (Beckman Instruments, Inc., Fullerton, Calif.). All samples were prepared in triplicate. Experiments with buffer containing 100 mM sodium citrate

\(^*\) Corresponding author.
20

KASHKET AND KASHKET

Infect. Immun.

instead of sodium acetate gave essentially the same results as those carried out with the latter system. Preliminary studies indicated that steady state was achieved rapidly. The uptake of radioactivity was unchanged between 5 (earliest point taken) and 30 min of incubation. Glycolytic rates of cells incubated under similar conditions were measured as described previously (15).

The radiolabeled compounds were purchased from New England Nuclear Corp., Boston, Mass.

RESULTS

ΔpH. The pH of four strains of glycolyzing streptococcal cells was found to be more alkaline than the medium at pH values ranging from 4.8 to 7.1 (Fig. 1). However, the ΔpH values decreased as the pH was reduced. At a medium pH about 7.0, the mean pH was about 7.6 (ΔpH = 0.6) (Fig. 1). Three of the strains had essentially the same pH, and only S. mutans IB 1600 maintained a lower pH (ΔpH = 0.29). At a medium pH of about 5.5, the mean pH was about 6.4 (ΔpH = 0.9).

The maintenance of the ΔpH was dependent on glycolysis, as seen in experiments with starved cells. Cells of S. salivarius ATCC 25975 were incubated at 37°C in buffer at pH 7.0 without glucose until endogenous energy sources were depleted, as shown by the cessation of acid production. The ΔpH was found to be close to zero. When glucose was added, the ΔpH increased to 0.53. For comparison, fresh, unstarved cells supplemented with glucose had a ΔpH of 0.55.

Effect of fluoride on ΔpH. We examined the effect of F⁻ on the ΔpH in oral streptococci with different sensitivities toward F⁻. The four strains tested, in decreasing order of F⁻ sensitivity, were S. salivarius ATCC 25975 (wild type), S. sanguis H7PR3, S. mutans IB 1600, and the F⁻-resistant mutant of S. salivarius, strain Flr103 (2, 15). The addition of F⁻ led to a reduction of the ΔpH of the F-sensitive microorganisms (Fig. 2). In cells of S. salivarius ATCC 25975 at pH 7.0, the ΔpH was reduced from 0.59 ± 0.04 (mean ± standard deviation; n = 2 to 5) (pH₁ = 7.64 ± 0.04) to 0.17 ± 0.05 (pH₁ = 7.23 ± 0.06) by 10 μg of F⁻ per ml (0.5 mM NaF) and was completely dissipated by 100 μg of F⁻ per ml (Fig. 2A). In contrast, the F⁻-resistant Flr103 cells were unaffected by F⁻ at concentrations up to 100 μg/ml.

The ΔpH in S. sanguis was 0.63 ± 0.01 (pH₁ = 7.66 ± 0.06), and addition of F⁻ brought about a progressive reduction of the pH gradient until, at 100 μg of F⁻ per ml, the ΔpH was 0.01 ± 0.24 (pH₁ = 7.09 ± 0.31). The ΔpH of uninhibited cells of S. mutans was 0.29 ± 0.01 (pH₁ = 7.27 ± 0.05) and remained unchanged even with 20 μg of F⁻ per ml. Addition of 100 μg of F⁻ per ml reduced the ΔpH to 0.13 ± 0.01 (pH₁ = 7.15 ± 0.01).

The effects of F⁻ on the ΔpH of all strains were more pronounced at pH 5.5 than at the higher medium pH (Fig. 2B). The ΔpH of strain Flr103 also was reduced by the addition of F⁻. At the lower concentrations of F⁻, the mutant strain was more resistant than the parent, but at 100 μg of F⁻ per ml, the ΔpH was almost completely dissipated.

When cells of S. salivarius ATCC 25975 and Flr103 were incubated with 100 μg of F⁻ per ml in buffers at different medium pH, we found that the mutant maintained the same pH₁ as either the parent or the mutant without F⁻ when the pH₂ was above 6.0 (Fig. 3). Below pH 5.8, the pH₁ of strain Flr103 dropped and the ΔpH of F⁻-treated cells was reduced essentially to zero. The curve for the parent incubated with 100 μg of F⁻ per ml was close to zero under all conditions of extracellular pH.

Eisenberg and Marquis (5) suggested that the entry of F⁻ (as HF) into the cells leads to the acidification of the intracellular environment. Accordingly, we examined the effects on cell-free preparations of these microorganisms of adding sufficient HCl to give H⁺ concentrations equivalent to those of the NaF used above. S. salivarius cells suspended in 0.15 M NaCl were sonicated with Ballotini glass beads for a total of 3 min and centrifuged at 10,000 × g for 10 min at 4°C to remove cell membranes and debris. The cell concentration (about 0.5 mg [wet weight] per ml) was the same as those used for the ΔpH assays. Addition of HCl up to 1.0 mM (equivalent to 20 μg of F⁻ per ml) reduced the pH by less than 0.08, not sufficient to account for the observed reduction of ΔpH in the intact cells. The buffering capacity
of the cell-free cytoplasm was exceeded after the addition of 5 mM HCl, although intact cells of strain Flr103 maintained the ΔpH unchanged at the equivalent concentration of F⁻ (100 μg of F⁻ per ml).

**Effect of fluoride on the Δψ.** It is possible that the Δψ may increase and compensate for the decrease of ΔpH through exchange of H⁺ for Na⁺ or K⁺ (18, 24). We measured the effect of F⁻ on Δψ and found that it was essentially the same in the parent and mutant strains of *S. salivarius* and was not affected by the change in medium pH from 7.0 (Table 1) to 5.5 (Table 2). Although Δψ remained unchanged in both strains after the addition of 100 μg of F⁻ per ml at pH 7.0, it was reduced significantly by F⁻ addition at pH 5.5. It is clear from these results that the ΔpH component was more sensitive to F⁻ addition than was Δψ and that Δψ did not increase to compensate for the reductions in ΔpH.

Δp, calculated by adding the ΔpH and Δψ components, was about 116 mV at pH 7.0 and 145 mV at pH 5.5 (averages for both strains) in the absence of F⁻. Fluoride reduced Δp in the parent at high and low buffer pH but did not affect Δp in the mutant at pH 7.0. Δp was not affected when glycolysis was inhibited by up to 25% (at either pH) but was reduced significantly when glycolysis was inhibited by 80% or more.

**DISCUSSION**

The oral streptococci maintained pH₃ higher than pH₄ at all values of buffer pH tested but, unlike other neupholies (24), failed to maintain pH₃ above 7.0 in the more acidic buffers. Responses to pH₃ resembled those reported for *S. lactis* (11) more than for *S. faecalis* (17, 24). Comparisons between the observed values of Δψ and those reported in the literature, however, are not as readily made. Kashket et al. (11) and Keevil and Hamilton (16) showed that the ionic nature of the medium affects Δψ, so that differences in the reported values of Δψ may reflect differences in the conditions of assay. It is important to note that, in the present study, Δψ was measured under identical conditions in all strains. The comparison between *S. salivarius* parent and mutant showed that Δψ was essentially the same in the two strains. Since both Δψ and ΔpH were unaffected in the mutant, it appears that the mutation to relative F⁻ insensitivity did not involve a change in regulation of Δp or its components. Furthermore, the present data indicate that insensitivity to F⁻ cannot be attributed simply to the exclusion of F⁻ from the cells, since the mutant strain has been shown to take up F⁻ at neutral pH (2), yet the ΔpH was not affected by F⁻ addition under these conditions.

In the present study evidence is presented that F⁻ addition leads to various degrees of dissipation of ΔpH in the oral streptococci, whereas Eisenberg and Marquis (5) had shown that the pH of the medium rises after the addition of F⁻ to *S. mutans* GS-5, consistent with a dissipation of ΔpH. However, the anti-metabolic effect of F⁻ on glycolysis is known to be greater at low than at high medium pH. The reason for this still is undetermined, but we propose that the differential effect of F⁻ in buffers at low and high pH reflects the steady-state pH₃ that is achieved after the rapid dissipation of the pH gradients. Thus, in cells exposed to F⁻ in neutral buffer, the final pH₃ will be close to neutral. Because of the shape of the known pH-response curves of glycolysis, the cells will be able to maintain a relatively high rate of glycolysis, and the percent inhibition of glycolysis will be low. However, in cells held in buffer at low pH₃, the pH₃ will drop to a level approaching that of the buffer, the glycolytic rate will diminish to a level appropriate for the low pH₃, and the percent inhibition will be greater than that at neutral pH₃. This is illustrated by the findings for *S. sanguis* H7PR3 incubated at pH 7.0 and pH 5.5 with 10 μg of F⁻ per ml. The ΔpH decreased by about 0.13 under both conditions (Fig. 2), so that the pH₃ dropped from 7.66 to 7.52 and from 6.20 to 6.08, respectively. Under these conditions, glycolysis was inhibited by 11% in neutral buffer but by 32% in buffer at pH 5.5 (15).

The present findings also suggest that growth of streptococci is limited when the pH₃ falls below a critical value. *S. salivarius* grows to a limiting pH of about 4.5, and it can be calculated that the pH₃ would be about 5.6 when the medium pH is 4.5 (Fig. 3). Maximal growth of the mutant, however, was only about 60% of that obtained with the parent, and the final pH of the medium was 5.6 (12). Calculations show that at a medium pH of 5.6 the pH₃ would be about 5.75. If these values are applied to the growth results, it can be seen that

---

**TABLE 1. Δp of S. salivarius strains at pH 7.0 (mean ± standard deviation for two to five experiments)**

<table>
<thead>
<tr>
<th>Strain</th>
<th>F⁻ concen (μg/ml)</th>
<th>59ΔpH (mV)</th>
<th>Δψ (mV)</th>
<th>Δp (mV)</th>
<th>p&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Inhibition of glycolysis (Cs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 25975</td>
<td>0</td>
<td>34.7 ± 2.2</td>
<td>83.7 ± 1.0</td>
<td>118</td>
<td>&lt;0.01, NS</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>-4.4 ± 7.3</td>
<td>93.1 ± 4.7</td>
<td>89</td>
<td></td>
<td>80</td>
</tr>
<tr>
<td>Flr103</td>
<td>0</td>
<td>28.8 ± 1.3</td>
<td>83.7 ± 0.5</td>
<td>113</td>
<td>NS, NS</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>28.8 ± 1.1</td>
<td>84.0 ± 1.8</td>
<td>113</td>
<td></td>
<td>25</td>
</tr>
</tbody>
</table>

* The 59ΔpH and Δψ values were compared by using the Student t-test. The first value applies to the 59ΔpH values with and without F⁻, and the second to Δψ values with and without F⁻. NS: Not significant.

---

**FIG. 3.** Effect of the external pH on the collapse of ΔpH in *S. salivarius* by 100 μg of F⁻ per ml. The intracellular pH₃ of *S. salivarius* ATCC 25975 (squares) or Flr103 (circles) were determined in buffers at different pH₃, without (open symbols) or with 100 μg of F⁻ per ml (closed symbols). Each value is the mean of 2 to 3 experiments, assayed in triplicate: SD = 0.01 to 0.19. The dashed line indicates ΔpH = 0.
TABLE 2. $\Delta p$ of S. salivarius strains at pH 5.5 (mean ± standard deviation for two to five experiments)

<table>
<thead>
<tr>
<th>Strain and $F^-$ conc (mg/ml)</th>
<th>$59\Delta pH$ (mV)</th>
<th>$\Delta p$ (mV)</th>
<th>$\Delta p$ (mV)</th>
<th>Inhibition of glycolysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 25975</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>$68.6 \pm 6.1$</td>
<td>$70.7 \pm 9.9$</td>
<td>139</td>
<td>$&lt;0.0005$, 0.025</td>
</tr>
<tr>
<td>100</td>
<td>$11.2 \pm 8.6$</td>
<td>$49.3 \pm 5.0$</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>FII103</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>$70.2 \pm 4.7$</td>
<td>$80.2 \pm 7.5$</td>
<td>150</td>
<td>$&lt;0.0005$, $&lt;0.0005$</td>
</tr>
<tr>
<td>100</td>
<td>$12.0 \pm 6.5$</td>
<td>$41.8 \pm 7.5$</td>
<td>54</td>
<td></td>
</tr>
</tbody>
</table>

* See Table 1, footnote a.

Both the parent and the mutant stopped growing at a similar pH. These findings suggest that a threshold pH exists, below which growth is limited. However, the actual position of the threshold in growing oral streptococci may be lower than is apparent from these calculations, since it has been found that the $\Delta pH$ is lower in growing than in nongrowing cells of S. lactis (11). A threshold internal pH has been described recently for other bacteria (1). The threshold pH may vary among different organisms, and species that grow to a low pH (e.g., S. mutans [27]) may do so because they tolerate a reduction of pH to a lower value than less acid-tolerant species.

ACKNOWLEDGMENTS

This study was supported by Public Health Service research grants DE-03917 from the National Institute of Dental Research to S. K. and grant PCM 80-01178 from the National Science Foundation to E. R. K.

We thank Randi J. Preman and Yu Ping Luong for assistance in these studies.

LITERATURE CITED


