Role of the Cell Membrane in pH-Dependent Fluoride Inhibition of Glucose Uptake by *Streptococcus mutans*

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The effect of pH on the fluoride sensitivity of glucose uptake by whole cells and glucose transport by permeabilized cells of *Streptococcus mutans* was compared. Whole cells exhibited a marked pH-dependent sensitivity to fluoride over the pH range 7.0 to 5.0. As the pH was decreased, fluoride sensitivity increased. In contrast, no significant effect of pH on the fluoride sensitivity of glucose transport (e.g., phosphorylation) by permeabilized cells energized with 2-phosphoglycerate was noted. The relative effect of pH on the fluoride sensitivity of whole cell glucose uptake and fermentation was similar. These data are consistent with the notion that the cell membrane is impermeable to the fluoride anion and that intracellular accumulation of fluoride depends on translocation of hydrogen fluoride across the membrane.

Streptococcus mutans is a colonizer of the human tooth surface and is associated with the development of dental caries in humans (5, 6, 15). This organism is noted for its aciduric, as well as its acidogenic, properties relative to those of other common plaque microorganisms (8, 9, 15). Development of dental caries is thought to depend on the acid demineralization of tooth enamel as a direct result of the acidic fermentative activities of *S. mutans* and other plaque microbes (15). Plaque pH varies from near neutral values in the resting state (no fermentable substrates available) to well below pH 5 during periods of exposure to fermentable substrates (15). Fluoride is a well-known cariostatic agent which is responsible for a major reduction in the prevalence of dental caries (2, 12). The principal cariostatic effect of fluoride is considered to depend on its incorporation into the enamel crystal structure with a resulting decrease in acid susceptibility of the tooth mineral (14). Fluoride also has inhibitory effects on numerous enzymes (17) and on the fermentative activities and viability of oral microbes, including *S. mutans* (for reviews, see references 7, 11, 13, and 16). The fluoride sensitivity of *S. mutans* sugar fermentation and growth is generally increased as the pH is decreased (1, 7, 11, 16). It has been suggested that the pH-dependent nature of fluoride sensitivity is based on the passive permeability of the cell membrane to HF and impermeability to fluoride anion (16). As the pH of the environment is decreased, the absolute concentration of HF is increased ($pK_a \sim 3.45$) and the pH difference across the cell membrane promotes accumulation of fluoride into the more alkaline cell cytoplasm. This model is supported by the observation that the intracellular accumulation of fluoride increases with the magnitude of the pH difference across the cell membrane of *S. mutans* (16). In light of these observations, we chose to investigate the role of the cell membrane in the pH sensitivity of fluoride inhibition of glucose uptake and transport by *S. mutans* over the pH range commonly encountered in plaque. If the model described above is correct, whole cell glucose uptake, but not glucose transport by permeabilized

cells, should exhibit pH-dependent fluoride sensitivity. We report here results that are consistent with the HF model.

MATERIALS AND METHODS

Bacteria. Bacterial strains (*S. mutans* BHT, 6715, GS-5, Ingbritt, and 10449) were stored, carried, and grown as described previously (4). Briefly, anaerobic cultures from early stationary phase were harvested by centrifugation and washed in 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM $MgCl_2$ (PBMg). Washed cells were then suspended to give ca. 330 $\mu g/ml$ (dry weight) and incubated for 30 min at 37°C to deplete cellular reserves, and then they were collected and washed on membrane filters (pore size, 0.45 μm , type HA; Millipore Corp., Bedford, Mass.). Cells were then suspended in one-half volume of the same buffer and stored on ice for up to 60 min before use.

Glucose uptake, transport, and fermentation. The washed cells described above were prewarmed to 37°C at a concentration of ca. 330 $\mu g/ml$ in PBMg at the desired pH and mixed with any other desired substances (see individual experiments) prior to the addition of [^{14}C]glucose (D-[U- ^{14}C] glucose, 200 μM at 3 mCi/mmol) at time zero. Glucose uptake by whole cell suspensions was estimated by spotting 50- μl samples on Whatman 3MM paper circles followed by immediate immersion, and later batch washing, in methanol (3). Permeabilized cells were prepared by freeze-thaw and toluene-acetone treatment as described previously (3). Transport of radioactive glucose by permeabilized cells was determined in the presence of 1 mM 2-phosphoglycerate (2PG) as the energy source. Samples (100 μl) were removed to DE81 paper circles (Whatman) and batch washed in ice-cold distilled water as before (3). Radioactivity associated with paper circles was determined in a Beckman LS30 scintillation spectrometer. Note that in all cases described above, pH was monitored and found not to change over the course of the uptake and transport experiments. Fermentation of glucose was monitored at room temperature with an Orion pH meter. Cells were suspended in 10 mM PBMg which was initially adjusted to pH 7.0. Glucose was added to give a concentration of 10 mM, and incubation was allowed

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FIG. 1. Effect of pH on the fluoride sensitivity of glucose uptake by *S. mutans* BHT. Washed cells were depleted of endogenous glycolytic reserves by incubation with 1 mM 2-deoxy-D-glucose for 10 min at 37°C. Following removal of 2-deoxy-D-glucose, cells were suspended in 1 mM PBMg at pH 7.0, then diluted into 50 mM PBMg adjusted to pH 7.0 (●), 6.5 (○), 6.0 (Δ), 5.5 (□), and 5.0 (×). NaF was added to give the indicated concentrations, and after a 5-min incubation [14 C]glucose was added. Rates of glucose uptake were estimated over 2 (pH 7.0) to 7 (pH 5.0) min. Results are the mean of two experiments and were normalized to the rates (nanomoles per minute per 330 μ g of cells) at each pH obtained in the absence of NaF: pH 7.0 (11); pH 6.5 (9.13); pH 6.0 (6.38); pH 5.5 (4.51); pH 5.0 (2.31).

to proceed for 5 min before the addition of a variety of concentrations of NaF.

RESULTS

The effect of pH on NaF inhibition of glucose uptake by whole cells of *S. mutans* was examined. Washed cells were first incubated with 1 mM 2-deoxy-D-glucose for 10 min to deplete cellular pools of glycolytic intermediates (4). The results (Fig. 1) demonstrate the marked effect of pH on the fluoride sensitivity of glucose uptake by the organism. Cells not treated with 2-deoxy-D-glucose also exhibited pH-dependent fluoride sensitivity (data not shown). The cell responses over the pH range 7.0 to 5.5 appeared to be parallel, whereas those at pH 5.0 suggest a somewhat decreased slope and, thus, perhaps a different mechanism of inhibition. This is more apparent from a closer examination of the data. The ratio of the fluoride concentration required to cause complete inhibition to that concentration required to just commence inhibition of uptake was between 4.9 and 7.1 over the pH range 7.0 to 5.5. In contrast, the ratio for cells at pH 5.0 was 24.6. As noted earlier, HF may be the membrane-permeable form of fluoride. If so, the magnitude of the pH dependency of the inhibition response in Fig. 1 should be reduced if fluoride is expressed as HF rather than the salt. This appears to be the case (Fig. 2).

If the membrane is a barrier to the entry of fluoride anion but allows HF to translocate into the cytoplasm, one would

predict that glucose phosphorylation by permeabilized cells would not exhibit a pH-dependent sensitivity to NaF inhibition. Cells of *S. mutans* were permeabilized with toluene-acetone treatment and incubated at a variety of pHs with a range of concentrations of NaF. [14 C]glucose phosphorylation was energized by the addition of 2PG. Note that the usual energy source used in transport studies with permeabilized cells is phosphoenolpyruvate. However, the principal target of fluoride (enolase) converts 2PG to phosphoenolpyruvate. Glucose phosphorylation by permeabilized cells energized with phosphoenolpyruvate is, therefore, resistant to fluoride, hence, our use of 2PG. Fluoride inhibition of glucose phosphorylation by permeabilized cells exhibited no significant pH dependence (Fig. 3).

The effect of fluoride on the fermentation of glucose by several strains of *S. mutans* was also examined (Table 1). Cell suspensions were allowed to commence fermentation of glucose and then were exposed to fluoride. The pH at which the first evidence of inhibition was discernible and the final pH of the suspensions was recorded. Note that with increased concentration of fluoride both the final pH and the pH of initial inhibition increased. Thus, as with the uptake of glucose by whole cells, glucose fermentation also exhibited increased sensitivity to fluoride at lower pH values. Although different in the absolute concentration of fluoride required to cause inhibition, both glucose uptake and glucose fermentation exhibited a similar relative pH-dependent sensitivity to fluoride (Fig. 4). In this case, the concentrations of fluoride associated with inhibition of the two processes at pH 7.0 were set at 100%. Fluoride concentrations that caused (i) the commencement of inhibition of glucose uptake or fermentation or (ii) 50% or (iii) complete inhibition of glucose uptake at other pH values were compared. Note that the responses were similar, suggesting that there is a common mechanism for the inhibition of the relatively

FIG. 2. Effect of HF on glucose uptake by *S. mutans* BHT. The data from Fig. 1 have been redrawn as a function of calculated HF present at pH 7.0 (○), 6.5 (Δ), 6.0 (□), and 5.5 (▽).

FIG. 3. Effect of pH on the NaF sensitivity of 2PG-dependent glucose phosphorylation by permeabilized *S. mutans* BHT. Permeabilized cells were mixed with phosphorylation buffer at pH 7.0 (○, ●, ■), 6.5 (◆), 6.0 (△, ▲), and 5.5 (□) (see text), and [14 C]glucose phosphorylation was monitored in the presence of the indicated concentrations of NaF and 1 mM 2PG. Data were normalized to the rates of transport in the absence of NaF at pH 7.0 (5.00 nmol/min). Rates of phosphorylation at pH 6.5, 6.0, and 5.5 were 92, 65, and 23%, respectively, of the rate at pH 7.0. The rate of glucose transport in the absence of added 2PG was less than 0.1 nmol/min. Different symbols at pH 7 and 6 represent independent experiments.

short-term glucose uptake experiments (less than 10 min) and the longer (up to 120 min) fermentation studies.

DISCUSSION

The data presented here are consistent with the notion that the pH-dependent fluoride sensitivity of *S. mutans* is based on the membrane permeability of HF and the membrane impermeability of fluoride anion over the pH range encountered in dental plaque. An earlier examination of fluoride

FIG. 4. Relative effect of pH on the NaF sensitivity of glucose uptake and fermentation by *S. mutans*. Data from Fig. 1 and Table 1 were normalized to the concentrations of NaF, at pH 7.0, required to just commence inhibition of glucose uptake (○) or fermentation (△) and to cause 50% (□) or complete (▽) inhibition of glucose uptake.

accumulation by resting cell suspensions of *S. mutans* at pHs 2, 4, 5.5, and 8.2 found that the magnitude of accumulation was proportional to the pH gradient across the cell membrane (16). As the pH gradient increased, fluoride accumulation increased. The time course of fluoride accumulation exhibited a rapid initial uptake (<1 min), followed by a rapid efflux over the next 5 min and a markedly reduced rate of efflux from 5 to 10 min. In many cases, fluoride accumulation at 5 and 10 min was similar. Our experiments with glucose uptake by whole cells employed a 10-min preincubation with fluoride prior to addition of [14 C]glucose. Thus, it is likely that near equilibrium of fluoride distribution had been reached by the time glucose was added to the cells. On addition of glucose, alkalization of the cytoplasm may occur as a result of metabolism, and one might expect some redistribution of fluoride. Over the pH range 5.5 to 8.2, however, the magnitude of fluoride redistribution may be small and account for at most a two- to threefold change in accumulated fluoride (16). Other factors that can influence the fluoride sensitivity of *S. mutans* are known and include (i) a lower pH gradient (and thus reduced fluoride accumulation) with increased external pH (10), (ii) a higher rate of sugar uptake and metabolism at elevated pH values (Fig. 1 [10]) compared with lower pH values, (iii) the effective reduction of the fluoride sensitivity of enolase-dependent glucose uptake and transport by the presence of a pool of glycolytic intermediates in metabolizing cells (4; G. Germaine and L. M. Tellefson, Infect. Immun., in press), (iv) possible decreased sensitivity of enolase to fluoride at low pH (1), (v) decrease of cytoplasmic pH by fluoride (10) with possible indirect effects on metabolism, (vi) the cationic environment (potassium versus sodium), in which potassium has been shown to promote fluoride inhibition of glycolysis at neutral pH (13), and (vii) growth conditions (for reviews, see references 8 and 13). Thus, the relationship between

TABLE 1. Effect of NaF on the fermentation of glucose by *S. mutans*^a

NaF (mM)	pH at beginning of inhibition (mean $\pm$ SD)	Final pH (mean $\pm$ SD)
0		4.57 $\pm$ 0.15
0.5	5.14 $\pm$ 0.26	4.84 $\pm$ 0.45
1.0	5.42 $\pm$ 0.24	5.33 $\pm$ 0.24
2.0	5.74 $\pm$ 0.27	5.64 $\pm$ 0.23
4.0	6.30 $\pm$ 0.18	5.98 $\pm$ 0.23
8.0	6.92 $\pm$ 0.04	6.42 $\pm$ 0.38
12.0	7.00 $\pm$ 0.00	6.92 $\pm$ 0.07

^a Washed cell suspension (ca. 330 μ g/ml [dryweight]) in 10 mM PBMg (pH 7.0) was incubated with 10 mM glucose for 5 min, followed by the addition of NaF. The progress of fermentation was monitored by measuring the pH. All incubations were done at room temperature. Data shown are from experiments with *S. mutans* BHT, 6715, GS-5, Ingbritt, and 10449.

fluoride and *S. mutans* and some other oral microbes is indeed complex.

Increased knowledge of (i) the basis of the effects of pH and other environmental factors on the relationship of fluoride with the oral microflora and (ii) the nature of the plaque environment may lead to more effective ways of delivering fluoride such that significant antimicrobial effects are achieved which further reduce the cariogenicity of *S. mutans*.

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