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Comparative Acid Tolerances and Inhibitor Sensitivities of Isolated F-ATPases of Oral Lactic Acid Bacteria

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pH activity profiles and inhibitor sensitivities were compared for membrane ATPases isolated from three oral lactic acid bacteria, *Lactobacillus casei* ATCC 4646, *Streptococcus mutans* GS-5, and *Streptococcus sanguis* NCTC 10904, with, respectively, high, moderate, and low levels of acid tolerance. Membranes containing F_1F_0 ATPases were isolated by means of salt lysis of cells treated with muralytic enzymes. Membrane-free F_1F_0 complexes were then isolated from membranes by detergent extraction with Triton X-100 or octylglucoside. Finally, F_1 complexes free of the proton-conducting F_0 sector were obtained by washing membranes with buffers of low ionic strength. The pH activity profiles of the membrane-associated enzymes reflected the general acid tolerances of the organisms from which they were isolated; for example, pH optima were approximately 5.5, 6.0, and 7.0, respectively, for enzymes from *L. casei*, *S. mutans*, and *S. sanguis*. Roughly similar profiles were found for membrane-free F_1F_0 complexes, which were stabilized by phospholipids against loss of activity during storage. However, profiles for F_1 enzymes were distinctly narrower, indicating that association with F_0 and possibly other membrane components enhanced tolerance to both acid and alkaline media. All of the enzymes were found to have similar sensitivities to Al-F complexes, but only F_1F_0 enzymes were highly sensitive to dicyclohexylcarbodiimide. The procedures described for isolation of membrane-free F_1F_0 forms of the enzymes from oral lactic acid bacteria will be of use in future studies of the characteristics of the enzymes, especially in studies with liposomes.

Dental caries continues to be a major oral health problem in developed countries and is becoming an increasing problem in developing countries (4). The disease can be related directly to acid production by bacteria in the dental plaque which forms on teeth. The organisms most implicated in caries are the mutans streptococci, particularly *Streptococcus mutans* and *Streptococcus sobrinus* (9). In addition, *Lactobacillus* organisms are thought to be cariogenic in certain locales, for example, deep pits or fissures in the teeth (3). *Actinomyces* organisms have been implicated in root caries, although their involvement may be mainly in initial plaque formation on exposed roots, while subsequent damage is caused by mutans streptococci. A distinguishing characteristic of cariogenic bacteria is a high level of acid tolerance compared with that of other plaque bacteria. These organisms are particularly damaging to teeth because they can carry out glycolysis to produce acid at low pH values and appear to be responsible for the fall in pH values to somewhat below 4.0 in cariogenic plaque after exposure to fermentable sugars. Since the aqueous solubility of dental enamel increases exponentially with decreasing pH value (17), exposure to these very acid conditions can result in enamel erosion that cannot then be repaired during the normal acidification-demineralization-alkalinization-remineralization cycle.

The relative acid tolerances of dental plaque bacteria appear to depend on the activities of F-ATPases able to transport protons out of cells in association with ATP hydrolysis and thereby to maintain ΔpH across the cell membrane with the interior alkaline relative to the environment (1). Although lactic acid bacteria do not appear to maintain a set cytoplasmic pH value near neutrality, they are able to develop ΔpH of as much as 1 pH unit in acid environments (7). The glycolytic system is relatively acid sensitive, and maintenance of ΔpH with the cell interior less acid than the environment is critical for glycolytic activity in acid environments. Glycolysis by organisms such as the mutans streptococci has been found previously (5) to occur at pH values well below the minima for growth. Thus, it appears that growth of the organisms in plaque occurs at higher pH values during alkalinization phases, while most of the damage leading to caries is caused by nongrowing bacteria able to carry out glycolysis in acidified plaque.

The acid tolerance of plaque bacteria appears to be related not only to levels of F-ATPase in specific organisms but also to the pH optima of the enzymes. Organisms which are more acid tolerant have ATPases with lower pH optima for activity, as described in this paper. This finding of differences in pH optima is somewhat surprising in view of current knowledge of the conserved nature of F-ATPases and the relationships among lactic acid bacteria. However, the differences appear to be important for the cariogenicities of the bacteria and for plaque ecology.

MATERIALS AND METHODS

Bacteria. *Lactobacillus casei* ATCC 4646, *S. mutans* GS-5, and *Streptococcus sanguis* NCTC 10904 were maintained in the laboratory by means of weekly subculture on agar plates of brain heart infusion medium (Difco Laboratories, Detroit, Mich.) and as frozen stocks at −70°C.

Membrane isolation. Cells for membrane isolation were grown statically in 5-liter batch cultures at 37°C in brain heart infusion medium supplemented with 2% (wt/vol) glucose and 20 mM DL-threonine. The initial pH value of the medium was adjusted to 7.0, and cells were harvested in the late exponential phase before the culture pH had fallen

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below 5.0. The cells were converted to osmotically sensitive forms by treatment with lysozyme and mutanolysin as described previously (1) and lysed in 1.0 M NaCl solution with 10 μg of DNase and 10 μg of RNase per ml. Membranes were pelleted by centrifugation at 31,000 × *g* in a Beckman JA-20 rotor (Beckman Instruments, Palo Alto, Calif.), washed, and stored as frozen pellets or in buffer at 4°C.

ATPase assays. ATPase activity was assayed in terms of phosphate release by the method of Fiske and SubbaRow (6) with reagents obtained from the American Monitor Corporation (Indianapolis, Ind.). Assay mixtures contained 5 mM ATP and 10 mM Mg^{2+} ($MgCl_2$). Specific activities were calculated as micromoles of phosphate released from ATP per minute per milligram of protein. Protein was assayed by the method of Lowry et al. (10) with bovine serum albumin as the standard for intact membranes and F_1 preparations. Detection of protein in detergent extracts was by means of the bicinchoninic acid method (Pierce Chemical Co., Rockford, Ill.) because of interference by the detergents in the procedure of Lowry et al.

F_1-ATPase isolation. Crude F_1-ATPase preparations were obtained by washing isolated membranes by the procedures described by Senior et al. (13). The membranes were washed twice with Senior's +PAB buffer (40 mM ε-amino-*n*-caproic acid, 6 mM 6-aminobenzamidine, 5 mM Tris HCl, 0.5 mM dithiothreitol, 10% [vol/vol] glycerol [pH 7.0]), which resulted in extraction of peripheral membrane proteins. The F_1 enzyme was released during multiple washes with the same buffer lacking 6-aminobenzamidine. The wash solution was concentrated approximately 6× in Centriprep concentrators (Amicon Corp., Danvers, Mass.). The enzyme preparations were stored at −4°C in the −PAB buffer of Senior et al. (13) with 10% glycerol added. All F_1 ATPases were relatively insensitive to the inhibitor dicyclohexylcarbodiimide (DCCD), with less than 10% inhibition even at millimolar levels of the agent.

F_1F_0-ATPase extraction. F_1F_0 ATPases were extracted into HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer according to the procedures described by Solios and Furst (14), with the following modifications. The protease inhibitors phenylmethylsulfonyl fluoride, 6-aminobenzamidine, and ε-amino-*n*-caproic acid were included in the extraction buffer at concentrations of 1, 6, and 40 mM, respectively. Glycerol was also included, at a concentration of 20%, vol/vol. The pH value of the extractant was adjusted to the approximate pH optimum for activity of each enzyme: 5.5 for *L. casei*, 6.0 for *S. mutans*, and 7.5 for *S. sanguis*. The volume of the extractant solution was set so that the final detergent/protein ratio would be approximately 5:1 (wt/wt) and the final detergent concentration would be approximately 1.0%, wt/vol.

Fluoroaluminate inhibition assays. Enzyme preparations were mixed with fluoride solution before the assay so that the final concentration of NaF was 5.0 mM. Some preparations were pretreated with 0.5 mM deferoxamine (Sigma Chemical Co., St. Louis, Mo.) to chelate heavy metals. Then solutions containing $AlKSO_4$ were added at the time of substrate addition.

pH activity profiles. For assays with the membrane-associated enzymes, isolated membranes were pelleted in microcentrifuge tubes and resuspended in a buffer containing 50 mM Tris-maleate at the desired pH values. For assays with isolated F_1 or F_1F_0, the enzymes in concentrated solutions were added to Tris-maleate buffer to yield 50 mM buffer at the desired pH values, which were checked for each assay condition with a glass electrode. The enzymes were incubated in the test buffer for 30 min before addition of substrate at the pH value of the enzyme preparation.

RESULTS

Isolation of F_1F_0 ATPase from membranes. Previous studies of F-ATPases of oral lactic acid bacteria have been carried out mainly with F_1F_0 enzymes associated with isolated membranes or with F_1 enzymes separated from the membranes and from proton-conducting F_0 sectors (1, 16). An intermediate form of the enzymes is that of F_1F_0 complexes extracted from isolated membranes by use of detergents. Such complexes are water soluble, in contrast to the enzymes of isolated membranes, but still contain hydrophobic F_0 sectors. Our attempts to isolate such complexes showed that nonionic detergents, such as Triton X-100 or octylglucoside, were the most effective of a range of detergents tested for extraction. The optimal concentration for release was around 1% (wt/vol) at a detergent-to-protein ratio (wt/wt) of approximately 3.5. However, nearly optimal release occurred over the range from 1 to 3%. Higher concentrations of detergent resulted in less apparent release, possibly because the enzyme was adversely affected by the detergent. Similar results were obtained with all three test organisms.

Ionic detergents such as cholate and deoxycholate were effective for disrupting membranes, as indicated by solubilization of protein, but active ATPase complexes were not obtained when they were used. However, three washings of isolated membranes with low-ionic-strength buffer containing 6 mM 6-aminobenzamidine prior to detergent extraction increased the specific activities of F_1F_0 complexes, presumably by selectively extracting other proteins. For example, average specific activities of Triton X-100-extracted F_1F_0 complexes from 6-aminobenzamidine-treated membranes of *L. casei* ATCC 4646, *S. mutans* GS-5, and *S. sanguis* NCTC 10904 were, respectively, 1.30, 0.57, and 0.41 U/mg of protein, compared with values of 0.44, 0.38, and 0.21 for enzymes from untreated membranes.

Extracted F_1F_0 complexes were about as sensitive to the ATPase inhibitor DCCD as were membrane-associated enzymes (Table 1). Generally, even at a high concentration of 1 mM, DCCD causes only about 60% inhibition of ATPase activities of membranes isolated from oral lactic acid bacteria (1). Isolated F_1 complexes were very insensitive to DCCD, and maximal inhibition was always less than 10%. The data presented in Table 1 indicate that complexes extracted with octylglucoside were somewhat less DCCD sensitive than those extracted with Triton X-100. However, for enzymes extracted with either detergent, the level of sensitivity to DCCD was indicative of F_1F_0 complexes.

The data of Table 1 also indicate that the extracted enzymes could be inhibited by fluoride but that levels of inhibition by 5 mM NaF were low, especially for the enzymes from *L. casei*. In the presence of 0.5 mM deferoxamine, which complexes with heavy metals, inhibition was much reduced or completely eliminated. However, addition of 50 μM Al^{3+} to the mixtures greatly enhanced inhibition by 5 mM NaF. This result suggests that Al-F complexes, probably mainly AlF_4^-, are inhibitory for isolated F_1F_0, as they are for membrane-associated enzyme or isolated F_1 enzyme (15). Thus, sensitivity to fluoride, or more correctly to fluoride–heavy-metal complexes, did not vary greatly among the types of isolated ATPases.

Stabilization of isolated enzymes with phospholipids. In contrast to membrane-associated enzymes, isolated F_1F_0

TABLE 1. Inhibition of detergent-extracted F_1F_0 ATPases by DCCD and fluoride

Strain	Extraction detergent	% Inhibition[a]			
		Of untreated ATPases		Of deferoxamine-treated ATPases[b]	
		By 1 mM DCCD	By 5 mM NaF	By 5 mM NaF	By 5 mM NaF + 50 μM $AlKSO_4$
L. casei ATCC 4646	Triton X-100	64 ± 17	22 ± 3	10 ± 8	77 ± 7
	Octylglucoside	30 ± 10	11 ± 6	−5 ± 10	68 ± 7
S. mutans GS-5	Triton X-100	69 ± 21	58 ± 16	−8 ± 16	95 ± 1
	Octylglucoside	60 ± 5	50 ± 8	23 ± 12	88 ± 10
S. sanguis NCTC 10904	Triton X-100	73 ± 10	42 ± 21	12 ± 6	69 ± 11
	Octylglucoside	55 ± 18	61 ± 16	29 ± 1	90 ± 8

[a] Means ± standard deviations; $n = 3$.
[b] Preparations were treated with 0.5 mM deferoxamine for 30 min to remove heavy metals prior to incubation with 5 mM NaF or 5 mM NaF plus 50 μM $AlKSO_4$. The final concentration of deferoxamine in the assay suspension was less than 100 μM.

complexes showed progressive losses in activity, generally after about 2 days of storage at 4°C. This loss did not appear to be due to proteolysis because, as shown for enzyme from *S. mutans* GS-5 by the data of Fig. 1A, addition of 1 mg of soybean phospholipid per ml markedly reduced loss of activity. Moreover, inactive enzyme could be reactivated by addition of soybean phospholipids (Fig. 1B). Similar results were obtained for each of the test organisms, although there was variability among various preparations even from a specific organism in how rapidly ATPase activities were lost without phospholipid supplements. Membrane-associated F_1F_0 enzymes and isolated F_1 enzymes were stable for weeks at refrigerator temperatures, and added phospholipids did not enhance their stabilities in storage.

pH activity profiles. In Fig. 2, pH activity profiles for membrane-associated ATPases, isolated F_1F_0 enzymes, and isolated F_1 sectors from *L. casei*, *S. mutans*, and *S. sanguis* are compared. The data for the membrane-associated enzymes show clearly the hierarchy of acid tolerance, with *L. casei* enzyme more tolerant than that from *S. mutans*, which is more tolerant than the enzyme from *S. sanguis*. These differences in tolerance reflect differences in overall acid tolerances of the organisms, for example, with respect to glycolysis by intact cells (11). The *L. casei* enzyme is clearly adapted to function best in acid conditions. Its optimal pH for activity is between 5.0 and 5.5, and it retains some 20% of full activity even at a pH value of 4.0. Activity decreased at pH values above the optimal range, and at a pH value of 7.0, the enzyme had only some 30% of its maximal activity.

In contrast, the membrane-associated enzyme of *S. sanguis* had a much higher pH optimum, close to 7.0, and was totally inactive at a pH value of 4.5. The enzyme of *S. mutans* was intermediate in its profile. The range of near-optimal pH values was broad, from about 5.5 to 7.5, and the enzyme retained some 40% of its maximal activity at a pH value of 5.0.

Most of the major differences among the enzymes from the different organisms were lost or greatly diminished when hydrolytic F_1 complexes were dissociated from membranes. These enzymes are fully active in hydrolyzing ATP at about the same rate as F_1F_0 enzymes (12), but hydrolysis is not coupled to proton translocation.

F_1F_0 complexes isolated by means of Triton X-100 extraction had pH activity profiles roughly similar to those of

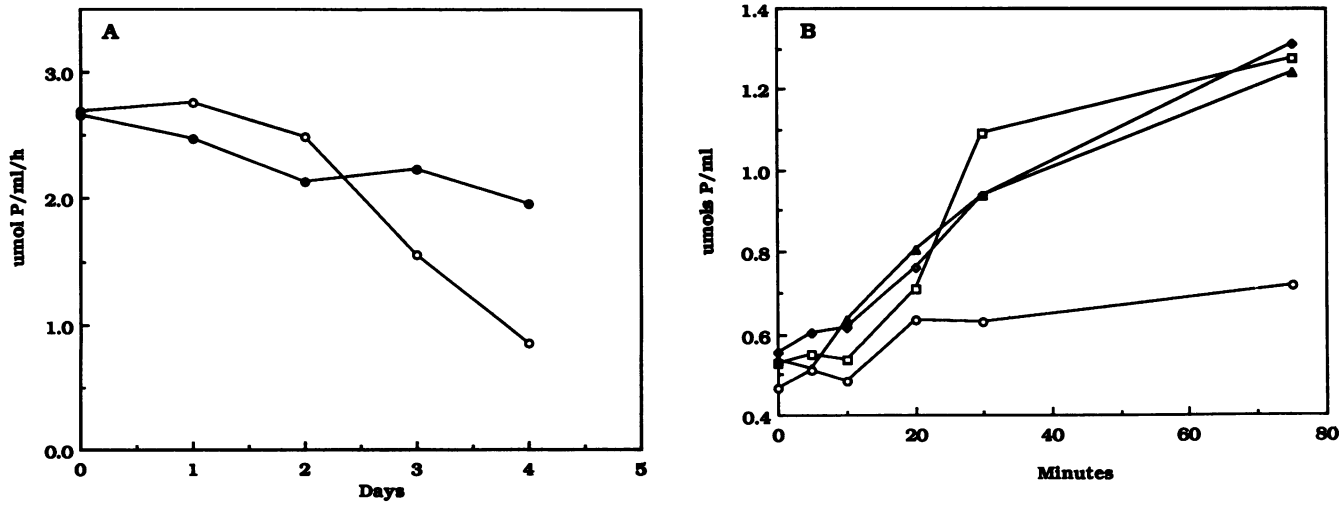

FIG. 1. Effects of soybean phospholipids on ATPase extracted from isolated membranes of *S. mutans* GS-5 with Triton X-100. (A) Loss of activity in preparations with (●) or without (○) added phospholipid (50 μg/ml); (B) reactivation of inactivated enzyme by phospholipid added to yield final concentrations of 0 (○), 50 (□), 100 (▲), or 200 (◇) μg/ml.

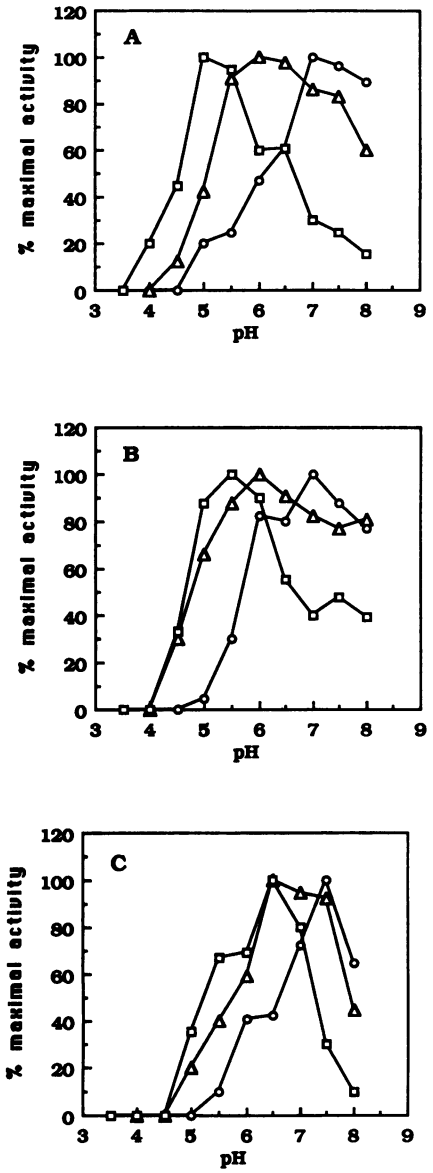

FIG. 2. pH activity profiles for ATPases of isolated membranes (A), Triton X-100-extracted enzymes (B), and F_1 complexes isolated by means of washing with low-ionic-strength buffers (C). The organisms used were *S. sanguis* NCTC 10904 (○), *S. mutans* GS-5 (△), and *L. casei* ATCC 4646 (□).

membrane-associated enzymes. The isolated complexes showed some apparent loss of acid tolerance, as, for example, for the enzyme of *L. casei*. However, the differences between isolated and membrane-associated enzymes were not great, and the pH activity profiles support the view that the isolated complexes were mainly F_1F_0 complexes.

DISCUSSION

In this paper, methods for detergent extraction of F_1F_0 ATPases from isolated cell membranes are described. The availability of membrane-free F_1F_0 complexes allowed us to distinguish between effects of general membrane association on pH activity profiles and those due to specific association with the F_0 sector. Isolated F_1 complexes freed from cell membranes are able to catalyze ATP hydrolysis uncoupled from proton translocation. However, their pH ranges for hydrolytic activity were narrower than those of membrane-associated F_1F_0 enzymes. Extracted F_1F_0 complexes had profiles similar to those of the membrane-associated enzymes. Thus, association of hydrophilic F_1 enzyme with hydrophobic F_0 sectors appeared to enhance tolerance to acid and alkaline conditions, but the integration of the complex into the cell membrane had relatively minor effects on pH activity profiles. The procedures described here for isolation of membrane-free F_1F_0 complexes will be useful in future studies of enzyme functioning, especially in studies with liposomes.

Overall, the results obtained indicate clearly that F-ATPases of oral lactic acid bacteria show molecular adaptations to the general acid-base physiology of the organisms. *L. casei* ATCC 4646 is the most acid tolerant of the three bacteria tested and can carry out glycolysis at pH values as low as 3.1. The low pH optimum of its F_1F_0 ATPase of about 5.0 is close to that of the membrane ATPase of the acidophile *Sulfolobus acidocaldarius* (8). *S. mutans* GS-5 is less acid tolerant and has a minimal pH value for glycolysis of about 3.4. The pH optimum for its F_1F_0 ATPase is about 6.0. *S. sanguis* NCTC 10904 is the least acid tolerant, with a minimum pH value for glycolysis of about 4.1. The optimal pH value for its F_1F_0 ATPase is close to 7.0.

F_1F_0 ATPases appear to be the major engines for maintaining ΔpH across the membrane for cells in acid environments with the internal pH alkaline relative to the external pH. Since the F_1 protrudes into the cytoplasm, its activity is affected primarily by the internal pH. The data presented here indicate that association of F_1 specifically with F_0 allows the enzyme to function over a wider pH range. The nature of the molecular differences among the enzymes that underlie differences in pH optima and activity ranges remains to be determined. However, it is clear that F_1F_0 ATPases of the more acid-tolerant oral lactic acid bacteria can function at lower cytoplasmic pH values, and the ability to continue to move protons out of the cell allows for functions such as growth and glycolysis to continue at lower external pH values. Minimum pH values for growth of oral lactic acid bacteria in complex media have been found previously (5) to be approximately 1 pH unit higher than minimum values for glycolysis. Moreover, environmental pH has been found previously (2) to have major effects on competitive growth of oral lactic acid bacteria. However, the minimum pH value for glycolysis is the pertinent value in regard to cariogenicity. Presumably, growth occurs in plaque mainly during alkalinizing phases at higher pH values, while enamel erosion occurs during acidification phases.

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