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ALTERED EXPRESSION OF THE H^+ ATPase IN STREPTOCOCCUS FAECALIS MEMBRANES

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SUMMARY. Evidence is presented that expression of the H^+ ATPase in S. faecalis is influenced by the extracellular pH and K^+ level during growth. Altered expression was detected by assay of F_1 ATPase and electrophoretic analysis of membrane proteins. K^+-limited growth caused about a 2-fold increase in the F_1 ATPase. The effect of growth at pH 6,7 and 9 was studied. Compared to cells grown at pH 7, growth at pH 6 increased the F_1 ATPase about 2-fold while growth at pH 9 reduced the F_1 ATPase by nearly 4-fold. The elevated F_1 ATPase activity in the pH 6 cells was associated with an increase in the F_1 ATPase α and β subunits in the membrane while the decrease in F_1 ATPase in the pH 9 cells was associated with a marked loss of the α subunit. It is suggested that intracellular protons may act as effectors which regulate expression of the F_1F_o gene cluster at the level of translation.

Some years ago it was suggested that the DCCD-sensitive membrane ATPase in S. faecalis, now referred to as F_1F_o or H^+ ATPase, is an inducible enzyme (1). It was found that K^+-limited growth of this organism induced about a 2-fold increase in the level of membrane associated ATPase activity. This effect was consistently reproducible and appeared to be fairly specific since there was no significant change in two other membrane-bound enzymes, p-nitrophenylphosphatase and NADH dehydrogenase. The cells grown under K^+-limiting conditions also exhibited a higher rate of glycolysis-dependent K^+ uptake as expected since K^+ uptake is coupled to the membrane ATPase activity (2). To explain the increased ATPase activity it was suggested that "...ATPase synthesis is fully derepressed when extracellular K^+ is low (0.4mM K^+ or less) and is repressed but only partially when external K^+ is high" (1). However, it was also recognized that the elevated ATPase activity could have been due to an unmasking of latent activity rather than derepression of de novo enzyme synthesis. Furthermore, subsequent investigations in other laboratories raised the possibility that bacterial membranes ATPases, other than the F_1F_o ATPase, might be responsible for the apparent induced ATPase activity. In studies of E.

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coli, Epstein and coworkers (3) found a membrane ATPase that is induced by K^+ deprivation but is quite different from the F_1F_o ATPase. Moreover, Hugentobler, et al. (4) have reported the isolation from S. faecalis membranes of a putative K^+ ATPase that is also structurally distinct from the F_1F_o ATPase. In addition, Heefner and Harold (5) found evidence for a Na^+ ATPase in S. faecalis.

It has been reported by Kobayashi, et al. (6) that the S. faecalis F_1F_o ATPase, by virtue of its function as a proton pump, serves to regulate the cytoplasmic pH. Here we present evidence that changes in the pH of the growth medium, as well as the K^+ level, alter expression of the F_1 ATPase. To obtain evidence for altered expression, changes in the amount of F_1 ATPase in the membrane were determined and correlated with membrane protein changes as determined by polyacrylamide gel electrophoresis.

METHODS

Growth Conditions - The organism used was Streptococcus faecalis (faecium) ATCC 9790. To test the effect of K^+-limited growth a previously described chemically defined growth medium (7) was used that was modified by increasing the concentration of each of the 20 L-amino acids to 2mM and doubling the level of purine and pyrimidine bases, vitamins, Mg^{2+}, Mn^{2+} and Fe^{2+}. In addition, K phosphate was replaced by 35mM Na_2HPO_4 and 42mM NaH_2PO_4; the glucose concentration was 55mM. The glucose stock solution was sterilized separately. This medium required the addition of K^+ to support growth (1).

A semidefined medium was used to study the effect of growth at an initial pH of 6,7 and 9. It contained 1% glucose, 1% tryptone (Difco), 0.5% yeast extract (Difco), 4.6mM K^+ (8) and 83mM phosphate added in the form of Na salts. Adjustment of the medium to pH 6,7 or 9 was carried out by addition of 0.1 volume of 830mM NaH_2PO_4 or Na_2HPO_4 or a 1:1 mixture of each. The glucose and Na phosphate solutions were sterilized separately.

For both the chemically defined and semi-defined medium growth was initiated by the addition of 50 ml of an inoculum culture, grown in the medium to be studied, to 450 ml of the same medium. Cell growth, at 38°, was monitored by optical density readings in a Klett photometer using a flask with a side arm. The cells were harvested near the end of log phase (about 3 cell generations) which took 3-5 hours, and washed twice with water.

Membrane Isolation - To isolate membranes the cells were first converted to protoplasts (9) by incubation for 45 min at 38° in 15 ml of a solution containing 5mM Tris Cl (pH 7.5), 0.5mM $MgCl_2$, 0.4M glycylglycine, 1 ug DNAase and 3mg lysozyme. The protoplasts were disrupted by metabolic lysis (9) initiated by addition of 300mM glucose and incubation for 5 min at 38°. The resulting membrane ghosts were centrifuged at 78,000xg for 10 min at 4° and washed by repeated centrifugation, once with 10mM $MgCl_2$ and 3 times with 2M LiCl-0.25M Tris Cl (pH 7.5) essentially as previously described (10) and finally suspended in 2.0 ml of 10mM $MgCl_2$. This membrane preparation was used for SDS-PAGE analysis and for solubilization of the F_1 ATPase by the low-salt aqueous wash procedure (see below).

Release of F_1 ATPase from Membranes - The F_1 ATPase was released by a low salt aqueous wash procedure (10,11) from the membrane by repeated centrifugation (78,000xg for 10 min at 4°) , twice with 2.0 ml of 33mM Tris Cl (pH 7.5) and 4 times with 2.0 ml of 1.0mM Tris Cl (pH 7.5). The solubilized F_1 ATPase activity in each wash was assayed by measuring the Pi liberated (12) after incubation of an aliquot

with 5mM NaATP, 5mM $MgCl_2$ in 100mM Tris Cl (pH 7.5) at 38°. A unit of ATPase activity is defined as the amount of enzyme that liberates 1.0 umol Pi per min. Essentially, all of the solubilized ATPase appeared in the 1.0mM Tris Cl washes. The identity of the solubilized ATPase as the F_1 ATPase was verified by SDS-PAGE analysis which showed the typical 5 subunit pattern (13).

SDS-PAGE Analysis of Membranes - Membrane samples were prepared for electrophoretic analysis by drying an aliquot of the membrane suspension (see above) containing the desired amount of protein under a stream of N_2. Protein was measured according to Lowry et. al. (14). The dried sample was dissolved in 20 ul of 1% 2-mercaptoethanol, 1% SDS and 10mM Na phosphate (pH 7) and heated at 100° for 3 min, 5ul of saturated sucrose solution mixed with bromphenol blue was added for layering the sample in the gel slot. Electrophoresis under dissociating conditions was carried out in a 2.5-27% polyacrylamide gradient slab gel (Isolab Inc.) containing 0.1M Na phosphate (pH 7.0), 0.1mM EDTA and 0.1% Na dodecyl sulfate (15) for about 20 hours at 25 volts at 20°. After fixing in 10% trichloracetic acid, the gel was stained overnight with Coomassie Brilliant Blue R-250 in methanol-acetic acid H_2O (5:1:5) and was destained in the same solvent.

RESULTS

Effect of K^+-Limited Growth - Previously it was shown that 0.4mM K^+ was barely sufficient to sustain the maximum growth rate of S. faecalis while 0.2mM K^+ supported growth at about 50% of the maximum rate (1). To determine the effect of K^+ restriction on synthesis of the F_1ATPase, cells grown in a chemically defined media containing 0.4mM and 0.2mM K^+ were compared to cells grown in excess K^+. Isolated cell membranes were prepared and the solubilized F_1ATPase, stripped from the membranes by the low-salt aqueous procedure, was assayed (see Methods). The results (Table I) show that K^+ restriction induced about a 2-fold increase in the amount of the F_1ATPase in the membranes. The result is in good agreement with numerous previous experiments in

TABLE 1

Effect of Growth Conditions on F_1 ATPase in S. faecalis Membranes[a]

Experiment	Growth Condition	F_1 ATPase (units/mg membrane protein)
I	0.4mM K^+	0.91
	4.0mM K^+	0.48
II	0.2mM K^+	1.37
	20.0mM K^+	0.59
III	pH 6	0.75
	pH 7	0.44
	pH 9	0.12

[a]A chemically defined medium was used for experiment I and II; a semi-defined medium was used for experiment III. F_1 ATPase refers to the enzyme released from isolated membranes by the low-salt wash procedure (See METHODS).

which the specific ATPase activity of the original unstripped membranes were assayed (1).

Effect of pH - In order to test the effect of pH on expression of the F_1 ATPase three batches of cells were grown in media with an initial pH of 6,7 and 9 prepared as described under Methods. The growth rate in pH 6 medium was about $\frac{1}{2}$ the rate in the pH 7 and pH 9 medium. The cell membranes were isolated and the amount of the F_1 ATPase stripped from the membranes by the low-salt aqueous wash procedure was assayed as described under Methods. The results (Table I) show that cells grown in the acidified growth medium (initial pH 6) contained about 2 times more F_1 ATPase than the pH 7 cells and about 6 times more F_1 ATPase than the pH 9 cells.

The electrophoretic profiles of the membrane proteins in the pH 6,7 and 9 cells were also compared (Fig. 1). Of the 8 different subunit components of the F_1F_o

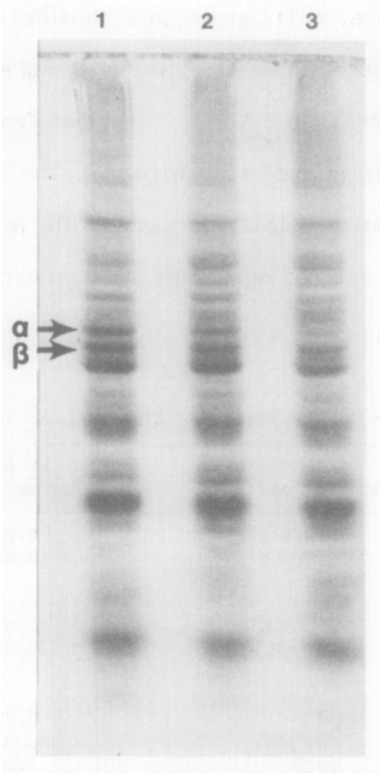

Fig. 1 - Effect of pH during growth on the electrophoretic profile of membrane proteins. The initial pH of the growth medium and the amount of sample protein were as follows: Lane 1: pH 6, 67 µg; Lane 2: pH 7, 75 µg; Lane 3: pH 9, 62 µg. Identification of α (55 kDa) and β (50 kDa) was based on co-migration with α and β in purified F_1 ATPase (16). See Table I for amounts of F_1 ATPase activity in the membrane.

ATPase (16) only the major subunits, α and β, belonging to the F_1 sector, were well resolved from the other membrane proteins. Inspection of the electrophoretic profiles reveals that there is more α and β in the pH 6 cell membranes than in the membranes from pH 7 cells (Fig. 1 lanes 1 and 2). However, while the pH 9 cell membranes had about the same amount of β as the pH 7 cells, the α subunit was nearly missing (Fig. 1, lane 3).

It should also be noted that the α:β ratio in the pH 6 and pH 7 cell membrane (Fig. 1, lanes 1 and 2) conforms to the characteristic 1:1 stoichiometry found in the isolated F_1 ATPase (16). However, because of the deficiency in the α subunit, the α:β ratio in the pH 9 cell membrane departs markedly from the normal 1:1 ratio (Fig. 1, lane 3). The observed changes in the amounts of α and β subunits in the membrane (Fig. 1) reflect qualitatively the level of the F_1 ATPase in the membrane as determined by assay of catalytic activity (Table I). The correspondence is not surprising since the α and β subunits are essential for catalytic activity (17). The observation that the α subunit is nearly absent in the pH 9 cell membranes (Fig. 1, lane 3) may have important implications with regard to the mechanism by which expression of the F_1F_o ATPase multigene cluster is regulated. (See Discussion).

DISCUSSION

The findings described here provide evidence that expression of the H^+ ATPase in S. faecalis (faecium) membranes is influenced by the extracellular K^+ level and the pH during growth. Two means were employed to detect altered expression of the enzyme. The first was the determination of the amount of the F_1 ATPase in the membrane as indicated by assay of the ATPase activity released from isolated membranes by the low-salt aqueous wash procedure. (10,11) (Table I). Solubilization of the membrane-bound F_1 ATPase by means of aqueous washes is a unique property of this enzyme and thus serves to distinguish it from other types of bacterial membrane ATPases (3,4). The other criteria for detecting altered expression was electrophoretic analysis of membrane proteins which revealed changes in the amount of F_1 ATPase subunit proteins, α and β (Fig. 1).

Assays of the F_1 ATPase stripped from the membranes showed that K^+-limited growth caused about a 2 fold increase in the amount of F_1 ATPase synthesized (Table

I). This result is in agreement with a set of 24 similar previous experiments (1) in which the ATPase activity of unstripped membranes was assayed and supports the view that K^+ limitation tends to derepress F_1 ATPase synthesis (1). Growth at different pH values had an even greater effect on F_1 ATPase synthesis than did K^+ restriction (Table I). Cells grown at pH 6 (initial pH) had about 6 times more F_1 ATPase than cells grown at pH 9 while cells grown at pH 7 had an intermediate level of F_1 ATPase (about 4 times more than the pH 9 cells) (Table I). One may conclude from the F_1 ATPase assays that increasing the pH tends to repress F_1 ATPase synthesis. This conclusion was substantiated by electrophoretic analysis of membrane proteins. The results showed that the increased F_1 ATPase activity induced by lowering the growth medium pH (Table I) was associated with an increased level of both the α and β subunit components of the enzyme in the membrane (Fig. 1, lanes 1 and 2). On the other hand growth at an alkaline pH (initial pH 9), which led to a marked decrease in F_1 ATPase activity (Table I), was associated with a marked loss of the α subunit but little change in β (Fig. 1, lane 3). The selective loss of the α subunit in the pH 9 cells results in a marked departure from the normal 1:1 subunit stoichiometry of α and β that is characteristic of the subunit stoichiometry of normal F_1 ATPase, $\alpha_3\beta_3\gamma\delta\varepsilon$, (13) and F_1F_o ATPase (16). It should be noted that in the pH 6 and pH 7 cell membranes the normal $\alpha:\beta$ ratio is apparently maintained. The results therefore suggest that the lowered level of the F_1 ATPase consequent to growth of cells at a highly alkaline pH may be due at least partly to decreased synthesis or to post-translational modification of the α subunit. There is evidence that the subunit stoichiometry of the E. coli F_1F_o complex encoded by the unc operon (17,18) is regulated at the level of translation (19). This suggests that the dramatic loss of the α subunit in the S. faecalis cells grown at pH 9 (Fig. 1, lane 3) might be due to an effect of an alkaline intracellular pH on translational control of the F_1F_o ATPase subunit stoichiometry.

The observed adaptive responses to the K^+ level and pH during growth (Table I, Fig. 1) are consistent with the physiological needs of the organism. First of all K^+ is absolutely essential for growth (1). Moreover, uptake of K^+ by S. faecalis under ordinary conditions depends on a protonmotive force generated by the F_1F_o

ATPase as well as ATP itself (2,20). On the other hand, the F_1F_0 ATPase seems to be dispensable in an enriched medium at an alkaline pH (21). Finally, there is evidence that the regulation of the cytoplasmic pH of S. faecalis, particularly in an acidic environment depends on the proton pumping activity of the F_1F_0 ATPase (6).

The altered expression of the streptococcal F_1F_0 complex described in this report suggests that K^+ and H^+ are regulatory effectors although H^+ has a much greater effect than K^+ (Table I). In this connection it is of interest that Kobayashi and coworkers (22) have recently reported that a low intracellular pH in S. faecalis during growth causes an increase in the ATPase activity of the membrane. They conclude that induction of increased membrane ATPase due to K^+ deprivation during growth (1) is a secondary consequence of the role of K^+ in regulating the intracellular pH. Therefore, it seems likely that intracellular protons may be the actual effector that regulates biosynthesis of the H^+ ATPase.

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