PROTONMOTIVE FORCE IN FERMENTING STREPTOCOCCUS LACTIS 7962 IN RELATION TO SUGAR ACCUMULATION

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SUMMARY: The fluorescent probe 1,1'-dihexyl-2,2'-oxacarbocyanine was used to measure the membrane potential in fermenting Streptococcus lactis 7962 cells. To calibrate the fluorescence changes and the membrane potential, the decrease in fluorescence of this dye was measured in valinomycin-treated cells and related to the membrane potential calculated with the Nernst equation at various K^+ ratios in/out. The membrane potential observed when cells ferment glucose or arginine was determined using such calibration curves. The protonmotive force during fermentation was calculated from the membrane potential and the pH gradient (measured by the distribution of 5,5-dimethyl-2,4-oxazolidinedione). The values obtained for the protonmotive force were found to correlate directly with the thiomethyl-β-galactoside accumulation in glucose- or arginine-energized cells. These data are in accordance with the chemiosmotic view of coupling of metabolic energy to the active transport of nutrients via electrochemical proton gradients.

We have shown previously (1) that in energy depleted Streptococcus lactis 7962 an imposed protonmotive force consisting of a membrane potential can drive the active transport of thiomethyl-β-galactoside (TMG). Valinomycin, the K^+-specific ionophore, was added to cells suspended in a K^+-free medium and the resulting efflux of K^+ generated a large membrane potential which, in turn, resulted in proton entry. Under these conditions there was a good quantitative relationship between the protonmotive force (membrane potential plus proton gradient) and the TMG accumulation, suggesting an obligatory coupling between proton and sugar uptake in this cell.

The purpose of the experiments presented in this report was to determine whether the protonmotive force is directly related to the accumulation of TMG by S. lactis 7962 cells under conditions where the energy for transport is supplied by glucose or arginine fermentation (2)

The membrane potential in these cells was measured with the fluorescent probe 1,1-dihexyl-2,2'-oxacarbocyanine (CC_6) which has been shown to monitor the membrane potential (3-5). When red cells (3,4) or phospholipid vesicles (5) were rendered permeable to K^+ ions by addition of valinomycin, the degree of fluorescence decrease of CC_6 or related dyes was found to be related to the medium K^+ concentration. When Streptococcus faecalis cells were mixed with the dye CC_6, addition of valinomycin or of glucose also resulted in a decrease in fluorescence (6). The fluorescent intensity decrease after valinomycin addition was again related to the K^+ concentration in the medium. The authors concluded that a membrane potential is generated in fermenting S. faecalis cells, as had been shown previously by Harold and Papineau (7) who observed the potential-dependent uptake of the cation dimethyl diphenyl ammonium (DDA^+), in the case of Na^+-loaded, K^+-depleted cells.

In order to use the decrease in fluorescence as a quantitative measure of the membrane potential in S. lactis cells, the fluorescence change was first calibrated by varying the membrane potential by altering the K^+ concentration ratios in/out in bacteria treated with valinomycin. The membrane potential calibrated by this method, as well as the pH gradient, were determined in cells fermenting glucose or arginine. The calculated protonmotive force (Δp) was compared to the TMG accumulation values.

Streptococcus lactis (ATCC 7962) cells were grown for 11 hours to early stationary phase as described previously (2), with 4 g KCl per liter instead of NaCl, and supplemented with 25 mM arginine. The cells were harvested, washed with 20 ml of 0.1M sodium phosphate buffer, pH 7.0, and resuspended in 10 ml of the same buffer. One tenth ml of saturated (about 5 M) glucose and 0.1 ml of 1 M KCl were added and the mixture incubated 30 min at 25 C. This incubation raised the internal K^+ from about 200 mM, typical of stationary phase cells, to

about 400 mM. The cells were centrifuged, washed with 20 ml of buffer, and resuspended in approximately 4 ml of buffer. A stock suspension was prepared by adding 20 µl of 3.5×10^{-3} M CC_6 dissolved in 95% ethanol to 4 ml of cells at a density of about 5300 Klett units (2). Aliquots of this stock suspension were diluted 1:30 for each determination. All manipulations, except for the initial centrifugations at 4 C, were carried out at 25 C. The buffer used throughout was 0.1 M sodium phosphate, pH 7.0.

When S. lactis 7962 cells were mixed with the probe CC_6 in sodium phosphate buffer, pH 7.0, and then treated with valinomycin, the fluorescence decreased in intensity (Fig. 1A); a maximum decrease of 25% was observed when no K^+ had been added to the medium (not shown). As the concentration of K^+ in the medium was raised, there was a proportionally smaller decrease in the fluorescence. In order to relate the membrane potential to the fluorescence change, the fluorescence at which no protonmotive force obtains was determined by treating the cells with gramicidin at the end of each experiment. This ionophore renders the membrane permeable to a variety of ions including H^+, Na^+ and K^+ (8), and abolishes TMG accumulation by S. lactis cells (unpublished data). In the presence of gramicidin the ions distribute themselves according to the Donnan potential, which occurs because of the presence of impermeable anions within the cell. Quantitative estimation of Na^+ and K^+ ratios after gramicidin addition confirmed this expectation. Calculating from either K^+ or Na^+ ratios in/out, the Donnan potential was found to be approximately 75 mV (external osmotic pressure = 260 mOsM). This value was unaffected by addition of glucose. In constructing a standard curve relating membrane potential to change in fluorescence it was found that the change after gramicidin addition was more reproducible within one batch of cells or from one experiment to another than the change obtained after valino-

mycin addition. For this reason all quantitative data refer to the values of the fluorescence change following addition of gramicidin.

Addition of either glucose or arginine to early stationary phase S. lactis cells resulted in fluorescence decrease (Fig. 1B). A

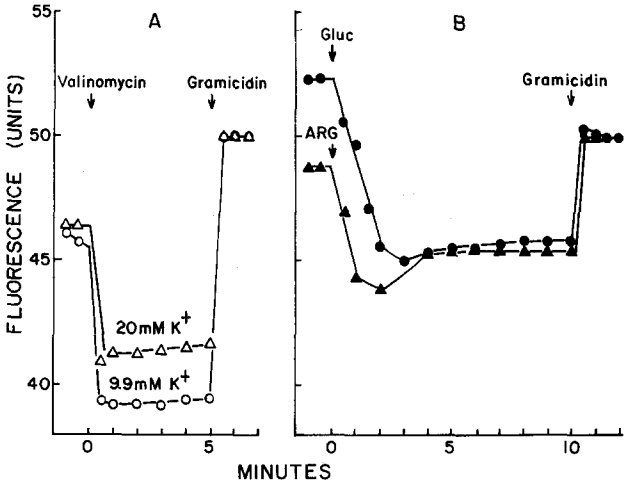

Figure 1: Fluorescence changes in S. lactis. Each cuvette contained 0.3 ml 1 M NaCl, 0.1 ml stock suspension of cells plus dye (described in text), sufficient KCl to give the indicated medium K^+ concentrations, and 0.1 M sodium phosphate buffer, pH 7.0, to a final volume of 3.0 ml. After 10 min valinomycin (5µl of 1 x 10^{-2}M in 95% ethanol), glucose (25µl of about 5 M), or arginine (50µl of 2 M) was added at 0 time, and gramicidin (5µl of 1 x 10^{-2}M in 95% ethanol) at 5 or 10 min, as indicated. The fluorescence was measured with an Amminco-Bowman spectrophotofluorometer at 450 nm excitation and 503 emission wave lengths and the readings normalized to final values of 50 arbitrary units.

standard curve was constructed for each batch of cells by relating the change in fluorescence to the membrane potential at two or more external K^+ concentrations. The $\Delta\psi$ values on the calibration curve (Fig. 2) corresponding to the fluorescence quenching obtained with glucose and arginine (Fig. 1B) were 35.5 mV and 39.0 mV respectively.

If there is an obligatory coupling between the transport of protons and TMG, then the protonmotive force should equal the force due to the TMG concentration gradient. The Δp (in mV) is the sum

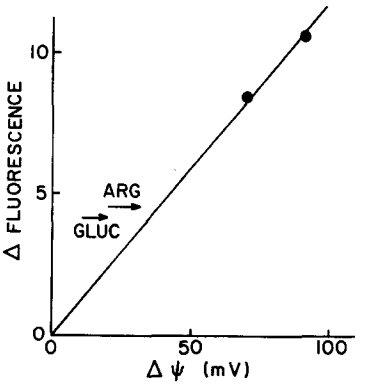

Figure 2: Relationship between the membrane potential ($\Delta\psi$) and fluorescence decrease. The Δ fluorescence is the value after gramicidin addition minus the value at 3-5 min after valinomycin addition, Fig. 1A. The $\Delta\psi$ was calculated from measured K^+ ratios in/out in parallel reaction vessels containing 0.3 ml stock suspension of cells plus dye, 0.9 ml 1M NaCl, KCl to give the indicated medium K^+ concentrations, 0.1 ml of 2mM either ^{3}H-D-sorbitol at 4 μCi/μmole or ^{14}C-D-sorbitol at 2.5 μCi/μmole, and 0.1M sodium phosphate buffer, pH 7.0, to a final volume of 9.0 ml. After 10 min valinomycin (15μl of 1 x 10^{-2}M in 95% ethanol) was added and four 1.0 ml aliquots were removed after 3-5 min. The cells were separated from the medium by Millipore filtration (1.2 μm pore size) without washing. Cell extracts were prepared by adding one drop of N-butanol to each filter and incubating in a boiling water bath for 15 min. Samples of 2 ml of the reaction mixture were centrifuged at 25 C. The cell extracts and supernatant fluids were assayed for K^+ concentration in a flame photometer after appropriate dilution. The K^+ concentration ratios in/out were calculated by subtracting the values for K^+ content of the contaminating extracellular fluid, whose volume had been determined in each sample from the content of ^{3}H- or ^{14}C-D-sorbitol. The cell density in the reaction mixture was 192 Klett units per ml (No. 42 filter), equivalent to 0.46μl of cell water per ml (2). The membrane potentials were calculated using the Nernst equation. Each point represents 4 determinations from duplicate reaction vessels. The arrows indicate the Δ fluorescence values obtained 8-10 min after glucose or arginine addition (Fig. 1B).

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of the electrical and chemical gradients for protons according to the equation (3):

$$\Delta p = \Delta\psi + 59\Delta pH$$

at 25 C, with ΔpH equal to the inside pH minus the outside pH; the force due to TMG accumulation, "Δp_{TMG}", in the same units is 59 log $[TMG]_{in}/[TMG]_{out}$. In the experiment illustrated (Table 1) glucose addition resulted in an 0.75 pH unit chemical gradient

TABLE 1

Energy Source	$\Delta\psi$ (mV)	Δ pH	Δp (mV)	$\frac{[TMG]_{in}}{[TMG]_{out}}$	$59\log\frac{[TMG]_{in}}{[TMG]_{out}}$
Glucose	35.5	0.75	79.9	16.1	71.2
Arginine	39.0	0.0	39.0	5.0	41.2

The values for $\Delta\psi$ are derived from Fig. 2. The pH gradient was determined from the distribution of 5,5-dimethyloxazolidine-2,4-[2-^{14}C]-dione, using ^{3}H-D-sorbitol to monitor the extracellular fluid space in each filtered sample, and calculated using the relationship described by Waddell and Butler (14). TMG accumulation was determined with 0.1 ml aliquots of stock mixture of cells and dye, as described previously (1).

(interior alkaline) and a 35.5 mV electrical gradient. The Δp calculated from the above equation was 79.9 mV. In this experiment TMG was accumulated 16.1 fold which, converted into the same units as the Δp, gives a value of 71.2.

When arginine was added as an energy source instead of glucose, no detectable pH gradient was observed. This is similar to S. faecalis cells which showed a pH gradient when fermenting glucose, but not arginine (10). The reason for a lack of pH gradient during arginine fermentation is unclear, but may be the result of the CO_2 produced (11). The protonmotive force calculated for arginine-energized cells was therefore 39.0 mV, which agreed well with the TMG accumulation (5-fold) value of 41.2.

These values and those from 6 other similar experiments are plotted in Fig. 3. The line is the theoretical one expected for a direct relationship between protonmotive force and galactoside accumulation. The experimental points fit well with the line. The lower three values were obtained with arginine fermenting cells, the upper five with glucose fermenting cells. Not shown are data obtained

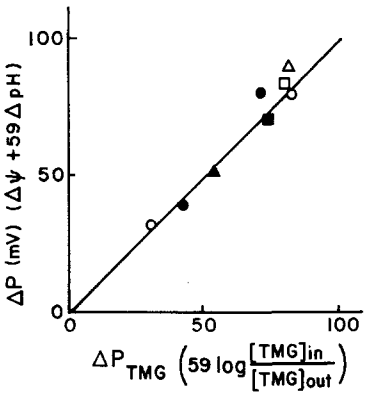

Figure 3: Relationship between protonmotive force (Δp) and TMG accumulation. Each symbol represents a separate batch of cells. The three lower points indicate arginine-energized cells, the upper five glucose-fermenting cells. The closed circles represent the experiments shown in Table 1. The line represents that expected from a direct relationship between Δp and TMG accumulation.

in the absence of added fermentable sugar: in that case there was no decrease in fluorescence, no pH gradient and no accumulation of TMG.

In previous experiments (1) in which TMG accumulation was energized by a membrane potential resulting from the efflux of K^+ in valinomycin-treated cells, there was discrepancy of about 25 mV between the Δp and the TMG accumulation values. It was proposed that the calculated $\Delta\psi$ values were too high because the concentration of K^+ in the periplasmic space was higher than that in the medium (which was less than 2 mM). In the present calibration experiments the medium K^+ concentrations were between 7 and 35 mM and no discrepancy between Δp and TMG accumulation was observed. This lends support to the proposed explanation for the previous results.

These data are consistent with the following sequence of events: fermentation of glucose or arginine by S. lactis cells results in the synthesis of ATP leading to extrusion of protons via the membrane-bound ATPase (12,13). The entry of TMG is, in turn, directly coupled to proton entry via the sugar transport protein. The driving force for

the entry of protons (protonmotive force) was found in these experiments to correlate directly with the capacity of the cells to accumulate the galactoside, in accordance with the chemiosmotic hypothesis of Mitchell (9).

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