Relationship between Intracellular Phosphate, Proton Motive Force, and Rate of Nongrowth Energy Dissipation (Energy Spilling) in *Streptococcus bovis* JB1

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Received 22 September 1997/Accepted 7 January 1998

When the rate of glucose addition to nongrowing *Streptococcus bovis* cell suspensions was increased, the fermentation was homolactic, fructose-1,6-diphosphate (FDP) increased, intracellular inorganic phosphate (Pᵢ) declined, and the energy-spilling rate increased. ATP and ADP were not significantly affected by glucose consumption rate, but the decrease in Pᵢ was sufficient to cause an increase in the free energy of ATP hydrolysis (ΔGᵢPᵢ). The increase in ΔGᵢPᵢ was correlated with an increase in proton motive force (Δp). *S. bovis* continuous cultures (dilution rate of 0.65 h⁻¹) that were provided with ammonia as the sole nitrogen source also had high rates of lactate production and energy spilling. When Trypticase was added as a source of amino acids, lactate production decreased; a greater fraction of the glucose was converted to acetate, formate, and ethanol; and the energy-spilling rate decreased. Trypticase also caused a decrease in FDP, an increase in Pᵢ, and a decrease in Δp. The change in Δp could be explained by Pᵢ-dependent changes in the ΔGᵢPᵢ. When Pᵢ declined, ΔGᵢPᵢ and Δp increased. The ratio of ΔGᵢPᵢ to Δp (millivolt per millimol) was always high (>4) at low rates of energy spilling but declined when the energy-spilling rate increased. Based on these results, it appears that Δp and the energy-spilling rate are responsive to fluctuations in the intracellular Pᵢ concentration.

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Materials and Methods

**Cell Growth.** *S. bovis* JB1 was routinely grown under anaerobic conditions at 39°C in basal medium containing (per liter) 292 mg of K₂HPO₄, 292 mg of KH₂PO₄, 480 mg of (NH₄)₂SO₄, 480 mg of NaCl, 100 mg of MgSO₄ · 7H₂O, 64 mg of CaCl₂ · 2H₂O, 500 mg of cysteine hydrochloride, 1 g of Trypticase (BBL Microbiology Systems, Cockeysville, Md.), and 0.5 g of yeast extract. The medium was adjusted to pH 6.7, and the final pH was never less than 6.5. Glucose was provided as the energy source at a growth-limiting concentration of 1 mg/ml (5.35 mM). *S. bovis* was also grown in glucose-limited continuous culture under O₂-free CO₂ at a dilution rate of 0.65 h⁻¹ (100-ml culture vessel, 39°C). Minimal medium contained 22 mM glucose, trace minerals, and vitamins (3) (yeast extract was omitted). Increasing amounts of Trypticase were added to the minimal medium as indicated in the figure legends. At least a 98% turnover of the medium through the continuous-culture vessel occurred between samplings (approximately 4 culture vessel volumes).

**Nongrowing Cells.** Exponentially growing cells were harvested and washed three times anaerobically in minimal medium lacking (NH₄)₂SO₄ (replaced by Na₂SO₄). Cell suspensions were placed in an anaerobic, water-jacketed (39°C) chemostat vessel (35 ml) that was purged with O₂-free CO₂. A pulse of glucose (1 mM final concentration) was used to energize the cells and reestablish ion gradients across the cell membrane. Glucose (1% [wt/vol]) was then added with an accurate peristaltic pump (model 2232, LKB Instruments, Inc., Gaithersburg, Md.) at a rate of 2 ml/h. Once the cell suspensions had equilibrated (30 min), samples (1 ml) were withdrawn at regular intervals. The removal of samples caused a decrease in volume and an increase in the rate of glucose delivery. By accounting for decreases in volume, glucose accumulation in the vessel, and cell protein concentration, it was possible to calculate the glucose consumption rate of nongrowing cell suspensions. This rate was verified by measuring the concentrations of fermentation acids.

**Intracellular FDP.** Batch cultures and cell suspensions having excess glucose were layered onto silicone for FDP extraction as previously described (3), but this procedure was too slow for glucose-limited cells. Glucose-limited cell suspensions and cultures (5 ml) were drawn into a syringe prefilled with 0.5 ml of 37% formaldehyde, mixed rapidly, and injected into a cold (stored on ice) 50-ml glass beaker. Preliminary work indicated that FDP concentrations were stable during the experiment.
even if the cells were left in the beaker for 5 min. The cell suspensions (1 ml) were then placed into a microcentrifuge tube containing 0.3 ml of silicone oil (equal-parts mixture of Dexter Hysol 550 and 560) layered on top of 0.1 ml of perchloric acid (0.1 mg of perchlorate plus 0.01 mg of methyl orange per ml). After centrifugation (13,000 × g, 5 min), FDP was assayed by a spectrophotometric assay as previously described (4). All determinations were performed in triplicate.

**Intracellular phosphate.** Intracellular phosphate also changed rapidly if glucose was limiting. The procedure for phosphate determination was similar to the one for FDP determination, except cells were centrifuged into 50 ml of perchloric acid. Cell-free supernatants and silicone oil were removed by vacuum, and the cell extracts were carefully resuspended in the perchloric acid and transferred to a fresh tube to avoid phosphate contamination from residual medium. Extracts were incubated on ice for 10 min and frozen (−215°C) until analysis. Phosphate was determined according to the method of Hess and Derr (10). The assay consisted of 10 to 20 μl of cell extract in a total volume of 600 μl of ammonium heptamolybdate, malachite green, and Sterox color reagent. Experiments to obtain standard curves used KH₂PO₄ in 10% perchloric acid (0 to 2,000 mM). Corrections were made for phosphate present in the extracellular space (medium concentration of intracellular phosphate, approximately 2.1 mM). New plastic vessels or acid-washed glassware minimized phosphate contamination. All determinations were performed in triplicate.

**Dp.** The pH gradient across the cell membrane and the electrical potential (Δφ) were determined by methods employing silicon oil centrifugation, the distributions of H⁻tetraphenylphosphonium bromide (H⁻TPP⁻) and ¹⁴C-benzoate across the cell membrane, and the Nernst equation (−2.3 RT/F × log ([concentration in]/[concentration out]), where RT is 2.59 kJ/mol and F is 96.5 kJ/V · mol). Intracellular volume was estimated from the difference between ¹⁴C-polyethylene glycol and ³H₂O distributions and was similar for growing and nongrowing cells (4.3 μl/mg of protein). Corrections were made for extracellular contamination. Nongrowing cell suspensions were incubated anaerobically at 39°C in a 35-ml vessel, and ³H-TPP⁻ and ¹⁴C-benzoate were injected directly into the vessel. Growing cultures were withdrawn from the continuous-culture vessel (190 ml), transferred anaerobically to a tube (2 ml) containing ³H-TPP⁻ and ¹⁴C-benzoate, and incubated at 39°C for 1 min. The pH gradient across the cell membrane and Δφ were dissipated by incubating the cells with a combination of nigericin (5 μM) and valinomycin (5 μM) for 10 min.

**RESULTS**

**Nongrowing cells.** When washed-cell suspensions of S. bovis JB1 were provided with a low rate of glucose addition via a peristaltic pump, extracellular glucose was never detected. By removing portions of the cell suspension, it was possible to increase the specific rate of glucose consumption by nongrowing cells in a stepwise fashion from 7 to 35 mmol of glucose/g of protein/h. ATP production was estimated from the production rates of fermenting cells in a stepwise fashion from 7 to 35 mmol of glucose/g of protein/h. Cell suspensions with glycolytic rates of less than 10 mmol of glucose/g of protein/h were heterofermentative (acetate, formate, ethanol, and lactate), but the fermentation was homolactic at higher rates of glucose consumption. ATP production was estimated from the production rates of fermen-
tation products (1 mol of ATP per mol of lactate in culture medium or 3 mol of ATP per 1 mol of acetate, 2 mol of formate, and 1 mol of ethanol).

When the glucose consumption rate increased, intracellular FDP increased from 2.5 to 17 mM and inorganic phosphate decreased from 45.5 to 7.5 mM (Fig. 1a). ATP and ADP concentrations increased slightly, but the ratio of ATP to ADP remained relatively constant (Fig. 1b). The intracellular pH was 6.7 ± 0.2. Based on the data of Rosing and Slater (21) and an intracellular magnesium concentration of 1 mM, it was possible to estimate the phosphorylation potential by using the formula

$$\Delta G_p = -285 \text{ mV} - 62 \log ([\text{ATP}]/[\text{ADP}] \times [\text{Pi}])$$

where \(\text{Pi}\) is intracellular phosphate. When the glucose consumption rate increased from 7 to 35 mmol of glucose/g of protein/h, the \(\Delta G_p\) increased from −410 to −460 mV (39 to 44 kJ/mol) (Fig. 2a). The \(\Delta p\) was also influenced by glucose consumption rate. At low rates of glucose consumption the \(\Delta p\) was only −90 mV, but rapidly glycolyzing cells had a \(\Delta p\) of −135 mV. The increase in \(\Delta p\) was due entirely to an increase in the membrane potential (\(\Delta \psi\)). The chemical gradient of protons was always less than −20 mV and did not change appreciably. The ratio of \(\Delta G_p\) to \(\Delta p\) was greater than 4 at low rates of glucose consumption, but this value decreased to 3.3 when the rate of glucose consumption was increased (Fig. 2b).

**Continuous culture.** When *S. bovis* was grown in continuous culture in a medium containing ammonia as the sole source of nitrogen at a dilution rate of 0.65 h⁻¹, all of the glucose was utilized, 95% of the glucose carbon could be recovered as either cells or fermentation products, and the cell yield was 15.3 g of protein/mol of glucose fermented. Lactate was the predominant end product, accounting for 85% of the glucose fermentation. The remaining products were acetate, formate, and ethanol (ratio of 1 to 2 to 1).

*S. bovis* could not utilize Trypticase as an energy source for growth, but Trypticase increased the cell yield of glucose-lim-
ined continuous cultures. The glucose yield increased from 15.3 to 30 g of protein/mol of glucose fermented, but some of this increase was caused by a shift from lactate production to acetate, formate, and ethanol production. This shift resulted in an increase in ATP production (Fig. 3a). Increased ATP availability could not explain all of the increase in glucose yield, however, and the ATP yield (or grams of protein per mole of ATP) also increased (Fig. 3b). When all changes in cell protein and ATP production were accounted for, cultures utilizing Trypticase as a nitrogen source decreased their specific rate of glucose consumption by 50% and their rate of ATP consumption by 65% (Fig. 3c), while maintaining the same growth rate.

The Trypticase-dependent decrease in the rate of ATP consumption was not correlated with a change in intracellular ATP or ADP (Fig. 4a), but there was a decrease in FDP and an increase in intracellular phosphate (Fig. 4b). Trypticase addition caused a decrease in the ΔGp of ATP hydrolysis (Fig. 5a), and most of this change was due to the change in intracellular phosphate (Fig. 4b). Δp also declined, and this decrease paralleled the decline in ΔGp (Fig. 5a). Virtually all of the change in Δp was due to a change in Dp, and the chemical gradient of protons was less than 20 mV. The ratio of ΔGp to Δp increased from 3.5 to 5.0 as Trypticase increased and the ATP consumption rate decreased (Fig. 5b).

DISCUSSION

It has long been noted that resting-cell suspensions had rates of catabolism higher than the rates needed for maintenance (24). Nongrowing S. bovis cells consumed glucose at a rate 10-fold higher than the maintenance rate, and this mechanism of energy spilling was constitutive (23). Based on the observation that nongrowth energy dissipation could be enhanced by protonophores and eliminated by an inhibitor of the membrane-bound ATPase, it appeared that S. bovis had a mechanism of cycling protons through the cell membrane (23). When glucose-limited continuous cultures were given a pulse dose of glucose, Δp (a driving force for proton influx) increased, but the relationship between Δp and energy spilling was not entirely clear (7).

Continuous cultures of S. bovis with low dilution rates had very low rates of nongrowth energy dissipation (high growth yield), but the growth yield of nitrogen-limited cells was abnormally low (3, 7). By using Stouthamer’s ATP requirements for bacterial growth (26), a maintenance rate of 5 μmol of ATP/mg of protein/h (3), and a dilution rate of 0.65 h⁻¹, it was possible to estimate the energy-spilling rate of growing cells in continuous culture. Previous work indicated that amino acid limitation (due to growth on ammonia nitrogen) increased the energy-spilling rate of S. bovis energy-excess batch cultures (22), and the present experiments indicated that amino nitrogen was also able to regulate the energy-spilling rate of energy-limited continuous cultures (Fig. 6). Other workers reported that bacteria growing in rich media had lower Δp values than bacteria growing in minimal media, but a relationship between Δp and energetic efficiency was not considered (12, 18). When S. bovis continuous cultures were supplemented with a source of amino acids (Trypticase), Δp and energy spilling both declined (Fig. 5a and 6).

The energy-spilling rates of growing and nongrowing S. bovis cells could be correlated with a decline in FDP and an increase in intracellular phosphate (Fig. 7). When intracellular phosphate increased, both the ΔGp and the Δp declined. Creation of the Δp is driven by the ΔGp, and some researchers have assumed that Δp is in equilibrium with ΔGp. However, the cell membrane is not a perfect insulator. If proton flux into the cell
The bacterial protein, colicin E1, is a Δp-dependent (voltage-gated) ion channel, with a threshold of approximately 80 mV (25). Further work is needed to see if S. bovis uses a similar mechanism to regulate membrane resistance and energy-spilling rate.

ACKNOWLEDGMENT

This research was supported by the U.S. Dairy Forage Research Center, Madison, Wis.

REFERENCES