Lactate Efflux-Induced Electrical Potential in Membrane Vesicles of *Streptococcus cremoris*

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We developed a procedure for isolating membrane vesicles from the homolactic fermentative bacterium *Streptococcus cremoris*. The membrane vesicles were shown to have a right-side-out orientation by freeze-etch electron microscopy and to be free of cytoplasmic constituents. The membrane vesicles retained their functional properties and accumulated the amino acids L-leucine, L-histidine, and L-alanine in response to a valinomycin-induced potassium diffusion gradient. Studies with these membrane vesicles strongly supported the possibility that there was a proton motive force-generating mechanism by end product efflux (Michels et al., FEMS Lett. 5:357–364, 1979). Lactate efflux from membrane vesicles which were loaded with L-lactate and diluted in a lactate-free medium led to the generation of an electrical potential across the membrane. The results indicate that lactate efflux is an electrogenic process by which L-lactate is translocated with more than one proton.

In previous publications, evidence was presented that lactate efflux from deenergized cells of *Streptococcus cremoris* and from membrane vesicles of *Escherichia coli* results in the generation of an electrochemical proton gradient ($\Delta\bar{\mu}_H+$) across the cytoplasmic membrane (18, 20). This electrochemical proton gradient can drive energy-requiring, membrane-bound processes such as solute transport, as has been shown for several amino acids (18, 20). Furthermore, growth studies with *S. cremoris* revealed that a decrease of the lactate gradient across the membrane resulted in a decrease of the maximal specific growth rate and cell yield. Further information about the mechanism of lactate efflux is difficult to obtain with whole cells because ATP hydrolysis can generate and energy-requiring processes such as ATP synthesis can consume the electrochemical proton gradient and consequently will interfere with lactate efflux or the lactate efflux-induced electrochemical proton gradient (17). For further studies, membrane vesicles are the model system of choice. Membrane vesicles of *S. cremoris* were obtained by a considerably modified version of the procedure of Kaback (9). Some structural and functional properties of these vesicles are described. In this membrane vesicle system, the generation of an electrochemical potential by lactate efflux could be demonstrated. These studies conclusively rule out an obligatory role of ATP hydrolysis for the generation of an electrochemical proton gradient in *S. cremoris*.

MATERIALS AND METHODS

Organisms and growth conditions. *S. cremoris* Wg2 was obtained from the Dutch Institute of Dairy Research (Ede, The Netherlands) and grown anaerobically on MRS broth (5) at a controlled pH of 6.3 in a 1-liter fermentor as described previously (14, 18).

Isolation of membrane vesicles of *S. cremoris*. Cells from a 1-liter culture were harvested in the late exponential growth phase (cell density, 0.4 to 0.5 g of cells dry [weight] per liter), washed with 150 ml of 100 mM potassium phosphate buffer (pH 7.0), and finally suspended in 10 ml of this buffer. The concentrated cell suspension was diluted with 20 ml of 100 mM potassium phosphate buffer (pH 7.0) containing 10 mM MgSO$_4$ and 150 mg of egg lysozyme (E. Merck AG, Darmstadt, Germany). The suspension was incubated for 30 min at 30°C. Subsequently, saturated K$_2$SO$_4$ was added to a final concentration of 0.15 M, which resulted in lysis of the cells. Immediately thereafter, the lysed cell suspension was diluted with 70 ml of 100 mM potassium phosphate buffer (pH 7.0) containing 50 μg of RNase (Miles Laboratories, Ltd., Slough, United Kingdom) per ml (final concentration) and 50 μg of DNase (Miles Laboratories, Ltd.), per ml (final concentration). This solution was incubated for 20 min at 30°C, K-EDTA (pH 7.0) was added to a final concentration of 15 mM, and incubation was continued for 10 min. After the addition of MgSO$_4$ (final concentration, 20 mM), the mixture was centrifuged (30 min, 48,200 × *g*, 4°C; first high spin). The pellet containing membranes, cells, and cell debris was resuspended in 25 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 10 mM MgSO$_4$. Whole cells and cell debris were removed by centrifugation (70 min, 750 × *g*, 4°C). The supernatant containing membrane vesicles

was carefully decanted. Membrane vesicles were collected by centrifugation (30 min, 48,200 × g, 4°C; second high spin). The bright-yellow pellet was resuspended in 50 mM potassium phosphate buffer (pH 7.0) containing 10 mM MgSO₄ to a concentration of 35 mg of membrane protein per ml. Aliquots of 0.1 ml were rapidly frozen and stored in liquid nitrogen until use.

Preparation of membrane vesicles of *S. cremoris* loaded with lactate. Membrane vesicles were prepared as described above, except that after the first high spin, the pellet was resuspended in 50 mM potassium phosphate buffer (pH 7.0) containing 50 mM potassium lactate (pH 7.0) and 10 mM MgSO₄. Subsequent steps were carried out in this buffer. After the second high spin, the pellet, containing purified membrane vesicles, was resuspended in the lactate-containing buffer and incubated for 1 h at room temperature. Finally, the lactate-loaded vesicles were concentrated (30 mg of membrane protein per ml) and frozen in liquid nitrogen.

Uptake of tetraphenylphosphonium (Ph₄P⁺) by lactate efflux. Lactate-loaded vesicles were quickly thawed and incubated for 2 h at room temperature. Samples of 2 μl were diluted in 200 μl of 50 mM potassium phosphate (pH 7.0) containing 10 mM MgSO₄ and 0.8 μM [³H]Ph₄P⁺ (2.5 Ci/mmol). Uptake was stopped by rapid dilution with 2 ml of 0.1 M LiCl. Uptake experiments were further performed as described previously (16, 19).

Potassium efflux-induced uptake of L-leucine, L-histidine, and Ph₄P⁺. To 0.1 ml of the concentrated vesicle suspension, valinomycin was added to a final concentration of 2 nmol per mg of protein. The vesicle suspension was incubated for 30 min on ice. Samples of 1 μl were rapidly diluted in 100 μl of 50 mM choline-phosphate buffer (pH 7.0) containing 10 mM MgSO₄, 4 μM valinomycin, and 28 μM L[U-¹⁴C]leucine (351 mCi/mmol), 29 μM L[U-¹⁴C]histidine (345 mCi/mmol), or 0.4 μM [³H]Ph₄P⁺ (2.5 Ci/mmol). Uptake measurements were performed as described above.

Enzyme assays. Spectrophotometric assays of α-glycerol-P dehydrogenase, fumarate reductase, NADH dehydrogenase, succinate dehydrogenase, malate dehydrogenase, lactate dehydrogenase, and aldolase were performed at 25°C with a double-beam spectrophotometer (Hitachi Perkin-Elmer, model 124, Tokyo Ltd., Japan). Aldolase activity was assayed by the method of Bergmeyer (2). Anaerobic α-glycerol-P dehydrogenase was assayed by phenazine methosulfate-mediated reduction of 3(4.5-dimethyl thiazolyl-2) 2,5-diphenyl tetrazolium bromide (MTT) at 570 nm as described by Kistler and Lin (10). The extinction coefficient of reduced MTT was taken to be 17/mmol per cm at 570 nm. Fumarate reductase activity was determined as described previously (3). NADH dehydrogenase, succinate dehydrogenase, malate dehydrogenase, and lactate dehydrogenase were determined by using dichlorophenolindophenol as the electron acceptor. The reactions were performed in Thurnberg cuvettes under a nitrogen atmosphere. Reaction mixtures (final volume, 1.0 ml) contained 50 mM potassium phosphate (pH 6.6), 10 mM magnesium sulfate, 0.1 mM dichlorophenolindophenol, and 0.04 mg of membrane protein. The reaction was started upon the addition of the substrate (final concentration, 0.1 mM). The extinction coefficient of oxidized dichlorophenolindophenol was taken to be 18.8/mmol per cm at 600 nm.

Menaquinone. Menaquinone was extracted from membrane vesicles by the method of Dunphy and Brodie (6) and determined by the method of Dadák and Krivánková (4).

Preparation of crude extracts of *S. cremoris*. Crude extracts of *S. cremoris* were prepared by three passages of a concentrated cell suspension (20 mg [dry weight] of cells per ml of 100 mM potassium phosphate, pH 7.0) through a French pressure cell (American Instrument Corp., Silver Spring, Md.) at an operating pressure of 20,000 lb/in².

Intravesicular volume. Intravesicular volume was determined from the distribution of [³H]water and [¹⁴C]gluconate by the procedure described by Bakker et al. (1). The intravesicular volume was 4.3 μl per mg of membrane protein.

Electron microscopy. Electron micrographs of freeze-etched replicas and thin sections of cells and membrane vesicles were obtained as described previously (8, 12).

Protein. Protein was determined by the method of Lowry et al. (15).

Materials. Radioactively labeled L[U-¹⁴C]leucine, L[U-¹⁴C]histidine, [U-¹⁴C]gluconate, and [³H]water were obtained from the Radiochemical Centre (Amersham, England). [³H]tetraphenylphosphonium was obtained from the Nuclear Research Centre (Beer Sheva, Israel). The uncoupler SF6847 (3,5-di-*tert*-butyl-4-hydroxybenzilidene malonitrile) was a gift from Y. Nishizawa, Sumitomo Chemical Co., Ltd., Osaka. All other reagents were of analytical grade and are commercially available.

RESULTS

Isolation and properties of membrane vesicles. To obtain membrane vesicles of *S. cremoris*, we developed a procedure based on the observations made by Kruse and Hurst (13). In this procedure, cells, harvested in the late logarithmic phase of growth, were incubated with relatively high concentrations of egg lysozyme (5 mg/ml). Cells lysed upon the addition of high concentrations of K₂SO₄ (0.15 M). The yield of membrane vesicles obtained by this procedure was low; usually 7 to 10 mg of membrane protein was obtained from 0.5 g (dry weight) of cells. The membrane preparation was essentially devoid of cytoplasmic constituents, as indicated by the presence of less than 1% of the aldolase activity present in crude cell extracts (11.6 μmol of fructose 1,6-diphosphate cleaved per mg of membrane protein per min).

Electron microscopy of membrane vesicles. Electron micrographs of thin sections of the membrane vesicles revealed closed sacs without recognizable internal structures (Fig. 1A). The maximum diameter of the membrane vesicles was 0.3 μm. To obtain information about the orientation of the membrane vesicles, we compared electron micrographs of replicas of freeze-etched membrane vesicles and whole cells. The convex face (inner fracture face) of the cytoplasmic membrane of whole cells was densely covered with particles (Fig. 1B), whereas the con-
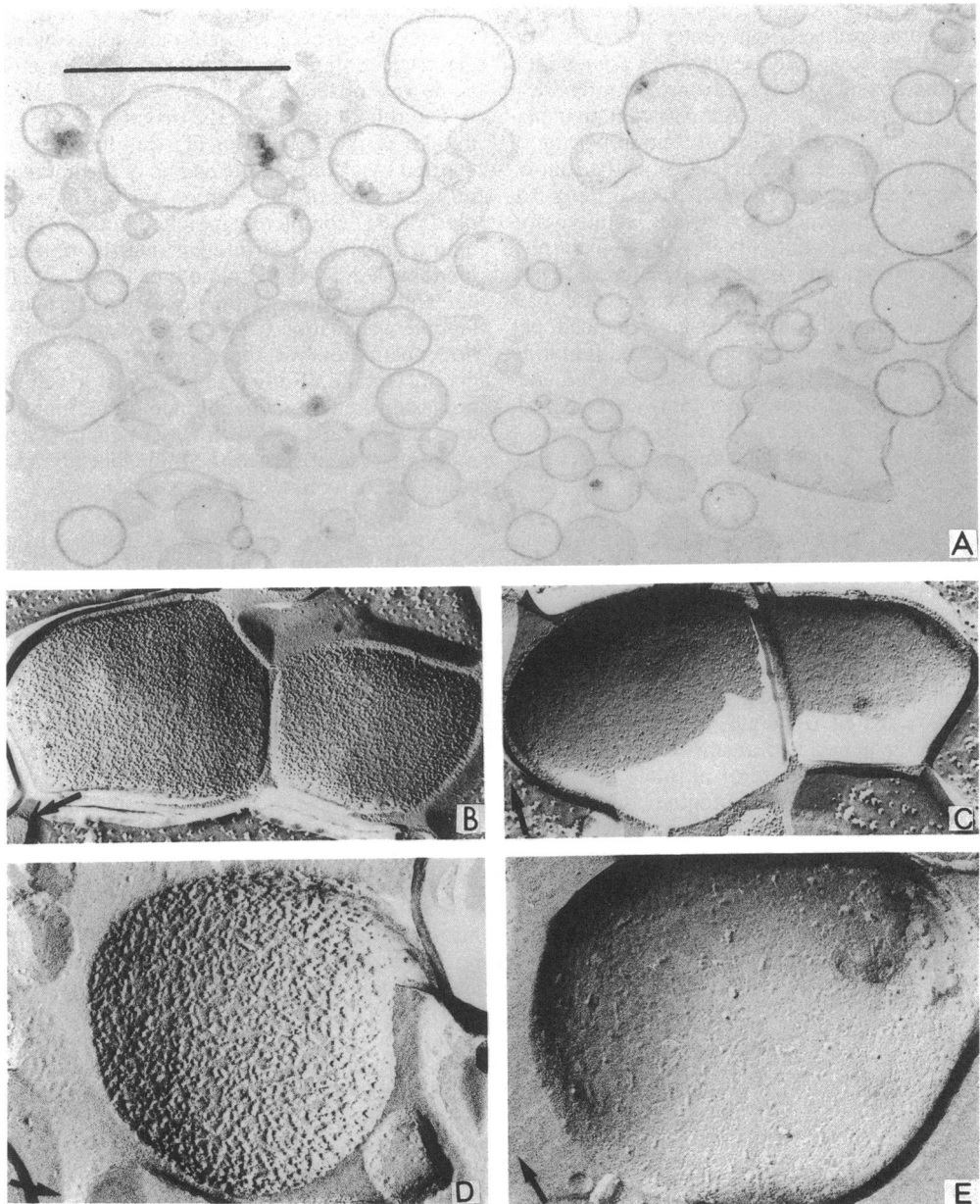
FIG. 1. Electron micrographs of intact cells and membrane vesicles of *S. cremoris*. A, Thin section of membrane vesicles of *S. cremoris* (×90,000). The bar represents 0.5 nm. B, Replica of freeze-etched cells of *S. cremoris* showing the convex inner fracture face of the cytoplasmic membrane, which is densely covered with particles (×52,000). C, Smooth concave outer fracture face (×52,000). D, Replica of freeze-etched membrane vesicles of *S. cremoris* showing the particle-rich convex membrane half (×111,000). E, Smooth concave membrane half (×111,000). Arrows indicate direction of the shadow.

cave face (outer fracture face) of whole cells had a low particle density (Fig. 1C). A similar distribution of the particles was observed in the inner fracture face and the outer fracture face of membrane vesicles (Fig. 1D and E, respectively). Of more than 100 replicas studied, not one

was observed with a different particle distribution. These observations strongly indicate that the orientation of the membrane vesicles is the same as that in whole cells.

Properties of membrane vesicles of *S. cremoris*. The internal volume of the membrane vesicles

was 4.3 µl per mg of membrane protein. Oxidized minus reduced difference spectra of reduced versus oxidized membrane vesicles indicated the absence of cytochromes and flavines. Membrane vesicles of *S. cremoris* contain menaquinone at a concentration of 9 nmol per mg of membrane protein. The activities of the following enzymes was determined: NADH dehydrogenase, succinate dehydrogenase, lactate dehydrogenase, malate dehydrogenase, α-glycerol-P dehydrogenase, and fumarate reductase. Only the activities of NADH dehydrogenase (2.8 nmol of dichlorophenolindophenol, reduced, per mg of membrane protein per min) and fumarate reductase (1.5 nmol of fumarate, reduced, per mg of membrane protein per min) were recorded, as the other enzyme activities were below the limit of detection (less than 1 nmol per min per mg of protein).

Potassium efflux-induced uptake of Ph₄P⁺ and amino acids. The observations presented above indicate that several essential elements of electron transport chains were missing in the cytoplasmic membrane of anaerobically grown cells of *S. cremoris*. The generation of a membrane potential, as indicated by the uptake of Ph₄P⁺, was not observed in the presence of an electron donor (we tested D-lactate, succinate, and formate) under aerobic or anaerobic conditions in the presence of fumarate or nitrate.

However, an electrical potential could be generated across the membrane of these vesicles by potassium efflux in the presence of valinomycin (Fig. 2B). Efflux of potassium from 75 mM internally to 0.75 mM externally resulted in the generation of a membrane potential ($\Delta\psi$) with a maximum value of –90 mV. This $\Delta\psi$ could drive

FIG. 2. A, Time course of K⁺ efflux-induced uptake of L-leucine and L-histidine. Membrane vesicles (30 mg of protein per ml) were incubated for 30 min on ice with 2 nmol of valinomycin per mg of protein in 50 mM potassium phosphate buffer (pH 7.0) containing 10 mM MgSO₄. The K⁺-loaded vesicles were diluted 1:100 in 50 mM choline-phosphate buffer (pH 7.0) containing L-leucine (□) or L-histidine (○) or into 50 mM potassium phosphate buffer (pH 7.0) (▼). B, Time course of K⁺ efflux-induced uptake of Ph₄P⁺. The K-loaded vesicles were diluted into choline-phosphate buffer (△) or potassium phosphate buffer (▲).

the uptake of L-leucine and L-histidine (Fig. 2A). In the absence of a potassium efflux-induced electrical potential, no uptake of these amino acids was observed.

The role of the $\Delta\psi$ as the driving force for the uptake of these amino acids was further documented by a study of the effects of the uncoupler on the uptake of these amino acids. The uncoupler SF6847 completely inhibited uptake of these amino acids. It is of interest that the uptake of histidine followed the uptake of Ph₄P⁺ (cf. Fig. 2A and B), whereas the uptake pattern of L-leucine was distinctly different. At this moment, no explanation for these observations can be given. Uptake of other amino acids driven by a potassium efflux-induced membrane potential was also studied. A low level of L-alanine accumulation was observed (12-fold), but no uptake could be demonstrated for L-glutamate, L-serine, and L-threonine. These studies show that the vesicles have retained their biological function with regard to transport of some amino acids.

Lactate efflux-induced electrochemical proton gradient. Dilution of membrane vesicles loaded with 50 mM potassium lactate into a lactate-free medium resulted in the uptake of the lipophilic cation Ph₄P⁺ (Fig. 3). The maximal $\Delta\psi$ observed was −55 mV. No uptake of Ph₄P⁺ was observed when lactate-loaded membrane vesicles were diluted into a medium containing 50 mM potassium lactate (Fig. 3). The lactate efflux-induced accumulation of Ph₄P⁺ was abolished in the presence of the uncoupler SF6847.

Dilution of potassium lactate-loaded vesicles into a lactate-free medium created also a small potassium gradient across the membrane (ca. 125 mM potassium inside and 75 mM potassium outside). This gradient, however, did not contribute to the generation of the lactate efflux-induced membrane potential, since uptake of Ph₄P⁺ was not affected by the presence of 25 mM K₂SO₄ in the dilution buffer. Moreover, no accumulation of Ph₄P⁺ was observed upon dilution of lactate-loaded membrane vesicles into a medium containing 50 mM choline-lactate.

DISCUSSION

Several procedures for the preparation of membrane vesicles of *Streptococcus* spp. have been described previously (7, 11). However, all of these procedures yield membrane vesicles which are heterogeneous with respect to the orientation of the membranes. The procedure described here yielded membrane vesicles of *S. cremoris* which were homogeneous in orientation.

Membrane vesicles isolated from *S. cremoris* have a number of properties in common with vesicles from *E. coli* (9) and *Bacillus subtilis*
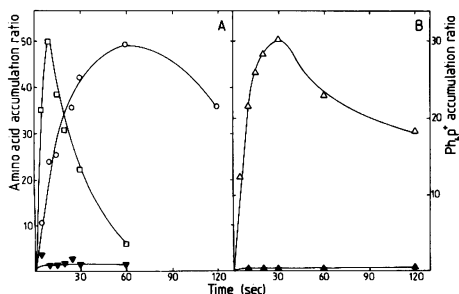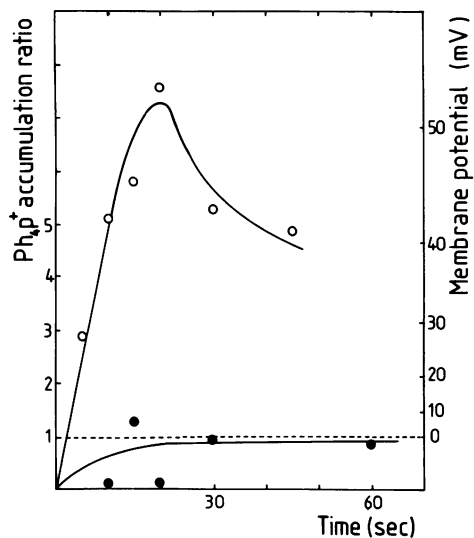
FIG. 3. Time course of L-lactate efflux-induced Ph_4P^+ uptake by membrane vesicles of *S. cremoris*. Membrane vesicles loaded with 50 mM potassium lactate were diluted 1:100 in 50 mM potassium phosphate buffer (pH 7.0) containing Ph_4P^+ (O) or into the same buffer supplemented with 50 mM potassium lactate containing Ph_4P^+ (●). The dashed line indicates the level of Ph_4P^+ accumulation when the internal concentration equals the external concentration.

(12). Membrane vesicles of *S. cremoris* are right-side-out oriented and devoid of cytoplasmic constituents. The average diameter is 0.1 μm, and the internal volume is 4.3 μl per mg of membrane protein. In contrast to vesicles from *E. coli* and *B. subtilis*, membrane vesicles of *S. cremoris* do not contain electron transfer systems. Of the known electron transfer carriers, only menaquinone is present in the cytoplasmic membrane of *S. cremoris*. No cytochromes or dehydrogenases (except for NADH dehydrogenase) were detectable. It is therefore not possible to generate an electrochemical proton gradient in these membrane vesicles by electron flow. Artificial methods have to be applied instead, such as valinomycin-mediated potassium efflux. By this procedure, an electrical potential is generated across the membrane. This electrical potential can drive the uptake of amino acids, as was demonstrated for L-leucine, L-histidine, and L-alanine. These observations demonstrate that the membranes of the vesicles of *S. cremoris* retained their property as a diffusion barrier.

Information about the role of lactate efflux in the generation of an electrochemical proton gradient was obtained with this model system. Lactate efflux from these membrane vesicles resulted in the generation of an electrical potential across the membrane. Since ATP is absent in these vesicles, ATP hydrolysis cannot contrib-

ute to the generation of an electrical potential. These observations therefore strongly support the energy recycling model of Michels et al. (17).

The generation of an electrical potential can only be accomplished if lactate is exported with more than one proton. The driving force for lactate (L^-) export is: $Z \log(L^-_{in}/L^-_{out}) + (n - 1) \Delta\psi - n Z\Delta pH$ in which $\Delta\psi$ is the electrical potential, ΔpH is the pH gradient across the cytoplasmic membrane, n is the number of protons transported in symport with L^- (lactate), and Z is 2.3 RT/F (R, gas constant; T, absolute temperature; F, Faraday constant).

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