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Light-induced generation of a protonmotive force and Ca^{2+}-transport in membrane vesicles of *Streptococcus cremoris* fused with bacteriorhodopsin proteoliposomes

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The light-driven primary proton pump bacteriorhodopsin has been incorporated in the cytoplasmic membrane of *Streptococcus cremoris*, in order to generate a protonmotive force across these membranes. This has been achieved by fusion of *S. cremoris* membrane vesicles with bacteriorhodopsin proteoliposomes. This fusion occurred when both preparations were mixed at low pH (less than 6.0), as shown by sucrose density gradient centrifugation and by dilution of fluorescent phospholipids incorporated into the bacteriorhodopsin proteoliposomes. Fusion was strongly enhanced by the presence of negatively charged phospholipids in the liposomal bilayer. When proteoliposomes were used that showed light-dependent proton uptake, the orientation of bacteriorhodopsin in the fused membranes was inside-out with respect to the in vivo orientation in *Halobacterium halobium*. Consequently, in the light a $\Delta\psi$, interior positive and a ΔpH, interior acid were generated. This protonmotive force could drive calcium uptake in the fused membranes. The uptake increased hyperbolically with increasing light intensity and was abolished by bleaching of bacteriorhodopsin. Addition of the ionophore valinomycin stimulated calcium uptake and led to an increase of the ΔpH. Calcium uptake was strongly decreased in the dark and in the light in the presence of uncouplers, nigericin or both valinomycin and nigericin.

Introduction

The electrochemical proton gradient or protonmotive force plays a central role in bacterial metabolism [1]. In bacteria, a protonmotive force can be generated by proton translocation from the cytoplasm to the external medium coupled to (i) electron transfer in electron-transfer chains, (ii) ATP hydrolysis via the membrane-bound Ca^{2+}, Mg^{2+} stimulated ATPase, (iii) efflux of metabolic endproducts and (iv) in Halobacteria by the light-induced proton pump bacteriorhodopsin. This protonmotive force is a driving force and a regulator of different metabolic processes, such as ATP synthesis, motility and secondary transport of solutes across the cytoplasmic membrane [1–3].

Lactic acid streptococci and a number of other fermentative bacteria lack functional electron-transfer chains [3]. These organisms are impaired

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Abbreviations: $\Delta\psi$, transmembrane electrical potential; ΔpH, transmembrane pH gradient; N-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; N-Rh-PE, *N*-(lissamine rhodamine-β-sulfonyl)dioleoyl phosphatidylethanolamine, DOPC, dioleoyl phosphatidylcholine; egg-PC, egg phosphatidylcholine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediamine tetraacetic acid; CCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; TPB^-, tetraphenylboron; TPP^+, tetraphenylphosphonium; DCCD, dicyclohexylcarbodiimide.

in the synthesis of porphyrins, and consequently cannot synthesize cytochromes [3,4] and are not able to generate a protonmotive force across their cytoplasmic membrane by electron transfer [5]. The organisms therefore rely on other processes, such as ATP hydrolysis [6] and endproduct efflux [7,8].

Membrane vesicles of bacteria are excellent modelsystems for the study of solute transport [9]. The orientation of the vesicular membrane is the same as the cytoplasmic membrane in intact bacteria. In the vesicular membrane, energy-transducing systems are present such as the Ca^{2+}- and Mg^{2+}-stimulated ATPases and the solute carrier proteins. All these systems have retained their functional properties and under appropriate conditions secondary transport of solutes can take place [9,10].

In membrane vesicles of lactic acid streptococci, it is difficult to generate a protonmotive force by ATP-hydrolysis, since ATP cannot pass the cytoplasmic membrane and thus cannot be hydrolysed by the Ca^{2+}, Mg^{2+} ATPase located at the inner surface of the vesicular membrane. Since electron flow also cannot be used to generate a protonmotive force, artificial methods have to be applied such as valinomycin-mediated potassium efflux. This procedure leads to the generation of an electrical potential across the membrane of *Streptococcus cremoris* which can drive the uptake of a number of amino acids [11].

The main problem with this procedure is that the electrical potential is transient which makes a detailed quantitative study of the role of the protonmotive force in solute uptake hardly possible. This urged us to search for a modelsystem in which a constant protonmotive force can be generated for a longer period of time. Membrane vesicles of *S. cremoris* fused with liposomes containing a primary proton pump can result in such a modelsystem. Recently, a method was described for the induction of fusion between liposomes and bacterial membrane vesicles [12]. In this study the pH-dependent interaction between large unilamellar phospholipid vesicles and membrane vesicles derived from *Bacillus subtilis* was investigated using a fluorescent assay for mixing of membrane phospholipids based on resonance energy transfer [13]. It was shown that efficient fusion occurs when liposomes, containing negatively charged phospholipids are mixed with the bacterial membranes at low pH. Fusion between the bacterial membrane vesicles and liposomes is protein-mediated, and is extremely sensitive to proteolytic treatment of bacterial vesicles [12].

Fusogenic properties similar to those described for *B. subtilis* membrane vesicles were found in cytoplasmic membranes of the other bacteria, including *Streptococcus cremoris* [12]. In this study we applied the low pH procedure to induce fusion between *S. cremoris* membrane vesicles and liposomes containing the light-driven proton pump bacteriorhodopsin [14]. We will show that *S. cremoris* membrane vesicles can indeed effectively be fused with bacteriorhodopsin proteoliposomes. The orientation of bacteriorhodopsin in the fused membranes is inside-out with respect to the in vivo orientation in *Halobacterium halobium*. Consequently, a $\Delta\psi$ interior positive and a ΔpH, interior acid, is generated in the light. The protonmotive force, generated by bacteriorhodopsin upon illumination, can drive calcium uptake in these fused membranes.

Materials and Methods

Growth of cells and preparation of membrane vesicles

Streptococcus cremoris Wg2 (prt^-) was obtained from the Dutch Institute of Dairy Research (Nederlands Instituut voor Zuivelonderzoek, Ede, The Netherlands). The organism was routinely maintained in 10% (w/v) skimmed milk and stored at -20°C. *S. cremoris* was grown anaerobically on MRS broth [15] at controlled pH of 6.4 in a 5 l fermenter. Membrane vesicles of *S. cremoris* were prepared as described previously [11], suspended in 50 mM potassium phosphate buffer (pH 7.0) containing 10 mM $MgSO_4$ at a protein concentration of 10–15 mg/ml and stored in liquid nitrogen.

Halobacterium halobium NRL (strain R1 [16]) was grown according to the method described by Danon and Stoeckenius [17]. Stationary phase cells were harvested by centrifugation and resuspended in distilled water containing pancreatic DNAase (10 μg/ml; type I; Sigma) and pancreatic RNAase (10 μg/ml; Sigma). The lysed cells were dialysed overnight at room temperature against distilled

water. Bacteriorhodopsin was purified by extensive washing (25 min; 38000 × *g*; 4°C) using alternately distilled water and 0.15 M KCl solution. Purple membrane sheets were resuspended in distilled water to a protein concentration of about 12 mg/ml and stored in liquid nitrogen.

Preparation of bacteriorhodopsin proteoliposomes

Purple membranes were resuspended in 50 mM potassium phosphate buffer, containing 0.1 mM ethylenediamine tetraacetate (EDTA) to a protein concentration of 4 mg/ml. Cardiolipin was added to the purple membranes at a concentration of 20 mg/ml (unless indicated otherwise). The suspension was subsequently sonicated with a 9 mm probe, at a frequency of 21 kHz and an amplitude of 4 μm (peak to peak) under nitrogen atmosphere for in total 700 s at 4°C. Alternating intervals of 15 s sonication and 45 s rest were used. Protein-free liposomes were removed by centrifugation (150000 × *g*; 60 min; 4°C). Bacteriorhodopsin liposomes were always used immediately after preparation.

Fusion between S. cremoris membrane vesicles and bacteriorhodopsin proteoliposomes

S. cremoris membrane vesicles (2–4 mg protein/ml) and bacteriorhodopsin proteoliposomes (1–2 mg protein/ml) were mixed at pH 5.0 in 25 mM potassium phosphate buffer supplemented with 25 mM sodium acetate. Membranes were mixed by rotation during 30 min at 4°C. Fused membranes were collected by centrifugation (48000 × *g*; 25 min; 4°C) and washed once in 50 mM potassium phosphate buffer (pH 7.0).

Sucrose density gradient centrifugation

The fused membranes were washed twice with 50 mM potassium phosphate buffer pH 7.0 to remove non-fused bacteriorhodopsin proteoliposomes and an aliquot of the resuspended pellet was mixed with 42% (w/v) sucrose in 50 mM potassium phosphate buffer pH 8.0 containing 1 mM EDTA to a final sucrose concentration of 7%. Subsequently, 1 ml of this suspension containing 2 mg protein was layered on top of a discontinuous sucrose gradient, composed of the following sucrose concentrations (w/v): 15% (96 ml), 30% 93 ml), 38% (3 ml), 42% (3 ml), 46% (3 ml), 50% (3 ml), 54% (3 ml) and 65% (3 ml) in 50 mM potassium phosphate buffer (pH 8.0) supplemented with 500 mM NaCl. After the addition of an overlay containing the phosphate buffer supplemented with 500 mm NaCl, the gradients were centrifuged in a Sorvall SS-90 vertical rotor at 34500 × *g* during 3 h at 4°C. During fractionation the gradients were scanned at 280 nm with a Perkin Elmer doublebeam spectrophotometer, model 124, using a flow cell. Absorption spectra in the visible region were recorded with an Aminco Change DW 2a spectrophotometer (American Instrument Company, Silver Spring, MD).

Fusion assay via fluorescence energy transfer

Fusion was monitored with the resonance energy-transfer fusion assay as described by Struck et al. [13]. For this assay bacteriorhodopsin (in purple membranes) was bleached by illumination with a 400 Watt lamp for 3 h in a thermostated vessel at 30°C in the presence of 2 M hydroxylamine [18] at a protein concentration of 1 mg/ml. After bleaching free hydroxylamine was removed by extensive washing with distilled water. Retinyl oxime was removed by freeze-drying of the preparation and several washings with hexane with brief sonication [19]. With this procedure more than 90% of the pigment was bleached as judged from the decrease in absorbance at 568 nm. The fluorescence donor *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (N-NBD-PE) and the fluorescence acceptor *N*-(lissamine Rhodamine-β-sulfonyl)dioleoyl phosphatidylethanolamine (N-Rh-PE) were both added to a concentration of 0.5 mol/%. Bacteriorhodopsin proteoliposomes (phospholipid/bacteriorhodopsin molar ratio, 160:10 were prepared as described above in 10 mM Hepes supplemented with 100 mM NaCl. Protein-free liposomes were removed by centrifugation (150000 × *g*; 60 min; 4°C).

Bacteriorhodopsin proteoliposomes (50 nmol phospholipid) were preincubated during 1 min in 2 ml 10 mM potassium phosphate buffer supplemented with 10 mM sodium acetate and 100 mM NaCl at the indicated pH at 25°C. Fusion was initiated by the addition of *S. cremoris* membrane vesicles (50 nmol phospholipid; 55 μg protein). Fusion of the vesicles leads to mixing of the lipids so that the fluorescence donor and acceptor dilute

in the plane of the newly formed membrane. This results in a decreased energy transfer of N-NBD-PE to N-Rh-PE which can be monitored as an increase in NBD fluorescence [13]. Continuous monitoring of NBD fluorescence was carried out with a Perkin Elmer MPF 44B spectrofluorometer equipped with a chartrecorder. NBD was excited at 475 nm and monitored at 530 nm with 5 nm bandpass slits. Following each measurement, vesicles were disrupted with Triton X-100 (1% final concentration). This treatment eliminates energy transfer and allows the determination of the maximal NBD fluorescence. The values were corrected for sample dilution and for the effect of Triton X-100 on the quantum yield of NBD-PE [13].

Centrifugation assay for membrane fusion

This assay was performed as described by Driessen et al. [12]. Bacteriorhodopsin proteoliposomes (phospholipid/ bacteriorhodopsin molar ratio, 160 : 1) containing a radioactive label or liposomes containing both a radioactive and fluorescent label were centrifuged in an Eppendorf microfuge for 5 min prior to fusion. After fusion and centrifugation the supernatant was used to assay the simultaneous disappearance of *S. cremoris* vesicles, bacteriorhodopsin, [^{14}C] egg PC, [^{14}C] cholesteryloleate and N-NBD-PE. To induce fusion equimolar concentrations of *S. cremoris* lipid and exogeneous lipid (0.5 mM) were incubated for 10 min at 37°C in 10 mM potassium phosphate buffer (pH 4.0), supplemented with 10 mM sodium acetate and 100 mM NaCl. Aliquots were taken to assay total amount of radioactivity, total amount of 7-nitro-2,1,3-benzoxadiazol-4-yl fluorescence and absorbance of bacteriorhodopsin at 568 nm. 7-Nitro-2,1,3-benzoxadiazol-4-yl fluorescence was measured with a Perkin Elmer MPF 44B spectrofluorometer (excitation, 475 nm; emission, 530 nm; and bandwidth, 5 nm) [13].

Measurement of the protonmotive force

The $\Delta\psi$ (interior positive) was calculated from the distribution of tetraphenylboron (TPB^-) between the bulk phase of the external medium and the intervesicular fluid using the Nernst equation. The concentration of TPB^- in the external medium was determined with a tetraphenylphosphonium (TPP^+) selective electrode [20] constructed according to Shinbo et al. [21]. A final concentration of 1 μM TPB^- was used. Measurements were performed in the presence of 0.1 μM TPP^+ to increase the permeability of the membrane for TPB^-. Potassium ion free buffers and membrane vesicles preparations were used. The intravesicular concentration was calculated from the amount of TPB^- which had disappeared from the external medium. Since TPB^- binding was not saturated between 1 and 30 μM TPB^-, an attempt was made to correct TPB^- accumulations for concentration-dependent binding, according to the model of Lolkema et al. [22]. Alternatively, the $\Delta\psi$, interior positive, was calculated from the distribution of thiocyanate (SCN^-) measured by rapid filtration. Since no binding of SCN^- was observed a correction for binding was not required. The $\Delta\psi$, interior negative, was calculated from the distribution of TPP^+, using a TPP^+ selective electrode as described above. TPP^+ was used at a concentration of 2 μM.

The ΔpH was determined from the uptake of [^{14}C]methylamine measured with automated flow dialysis, as described previously [23]. Measurements of pH changes were performed in a 2.0 ml thermostated incubation vessel (25°C) equipped with a magnetic stirrer. The pH of the medium was continuously measured with an Orion pH electrode ($pK = 2000$) connected to an amplifier and a recorder. Light from a slide projector (400 Watt) was provided to the membrane vesicle suspension by means of a fiber-optic light guide. Measurements were performed at a bacteriorhodopsin concentration of 0.25 mg protein/ml in 0.15 M KCl (pH 6.0). The pH changes upon illumination were calibrated by the addition of small amounts of 0.01 m KOH.

Transport assays

Uptake studies of calcium and thiocyanate were performed with the filtration technique [9,24]. Fused membranes were resuspended into 100 μl 50 mM potassium phosphate buffer, containing 10 mM $MgSO_4$ (pH 7.0) unless indicated otherwise. The suspension was stirred and illuminated for 2 min prior to the addition of 500 μM $^{45}CaCl_2$ or 20 μM [^{14}C]KSCN. Other additions were as indicated in the legends to the figures. At the times indicated

uptake was stopped by addition of 2.0 ml cold 0.1 M LiCl. The mixture was subsequently filtered over cellulose nitrate filters or for $^{45}CaCl_2$ over cellulose acetate filters with 0.45 μm pore size. In the latter case filters were presoaked in 0.5 mM $CaCl_2$ to decrease binding of calcium to the filters. The membrane vesicles were illuminated with white light with an intensity of about 1 $kJ \cdot m^{-2} \cdot s^{-1}$. The final protein concentration in all transport experiments was between 1 and 2 mg/ml. These experiments were done at 25°C. Radioactivity was determined with a liquid scintillation counter. The internal concentration of solutes accumulated by the vesicles was calculated using a value of 4.3 μl of intravesicular fluid per mg of membrane protein [11], unless stated otherwise.

Protein and lipid determination

Protein was measured by the method of Lowry et al. [25], using bovine serum albumin as a standard. Lipid concentrations were determined by analysis of lipid membrane phosphorus [26].

Materials

[^{14}C] methylamine (2.22 TBq/mmol), [^{14}C] KSCN (2.22 TBq/mmol) and [^{45}Ca]-calciumchloride (1.85 TBq/mmol) were obtained from the Radiochemical Centre, Amersham, U.K. N-NBD-PE, N-Rh-PE (both from Avanti Biochemicals, Birmingham, AL), [^{14}C] egg PC (2.5 TBq/mmol) and [^{14}C]cholesteryloleate (2.0 TBq/mmol) were generous gifts of Dr. J. Wilschut (Department of Physiological Chemistry, University of Groningen, The Netherlands). Egg PC and bovine heart cardiolipin were obtained from Sigma Chemical Co. Ionophores and uncouplers were dissolved in pure ethanol. Addition of these compounds were made so that the final ethanol concentration in the transport assay did not exceed 1% (v/v). All other chemicals were reagent grade and obtained from commercial sources.

Results

Fusion of S. cremoris membrane vesicles and bacteriorhodopsin proteoliposomes

Bacteriorhodopsin proteoliposomes can be prepared from purified lipids and purple membranes. Depending on the reconstitution method used, these bacteriorhodopsin proteoliposomes either alcalify [14,27] or acidify [14,28] their suspending medium. In this study we applied the sonication procedure for the preparation of bacteriorhodopsin proteoliposomes. Co-sonication of a suspension of synthetic phospholipids and purple membranes (phospholipid/bacteriorhodopsin molar ratio, 160:1) for 700 s resulted in a preparation that alcalified its surrounding medium upon illumination with a maximal extent of about 1.8–2.2 mol H^+/mol bacteriorhodopsin (data not shown). These values are similar to those reported earlier [14].

In order to test membrane fusion between *S. cremoris* membrane vesicles and bacteriorhodopsin proteoliposomes we applied the resonance energy transfer fusion assay as described by Struck et al. [13]. This assay monitors changes in the spatial organization of two non-exchangeable fluorescent lipid probes, the donor *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (N-NBD-PE) and the acceptor *N*-(lissamine Rhodamine β sulfonyl)phosphatidylethanolamine (N-Rh-PE). Dilution of both probes into a non-labeled membrane can be monitored as an increase in NBD fluorescence, due to the decrease in the energy-transfer efficiency between the two fluorescent probes.

Since the absorption spectrum of bacteriorhodopsin overlaps the emission spectrum of NBD, purple membranes had to be bleached in order to be able to use the resonance energy transfer assay. Bleaching of bacteriorhodopsin in the presence of 2 M hydroxylamine [18] and extraction of the retinyl oxime from the freeze-dried bleached purple membranes with hexane [19] resulted in more than 90% bleaching of the pigment. Bleached bacteriorhodopsin was incorporated into liposomes of various phospholipid compositions containing 0.5 mol % of both N-NBD-PE and N-Rh-PE. At this probe concentration the energy transfer efficiency was approximately linearly related to the N-Rh-PE probe concentration in the vesicular membrane [13,27]. Protein-free liposomes were removed by centrifugation.

Mixing of equal quantities (phospholipid) of *S. cremoris* membrane vesicles with bacteriorhodopsin proteoliposomes, containing cardiolipin, at low pH resulted in a decrease of the resonance energy

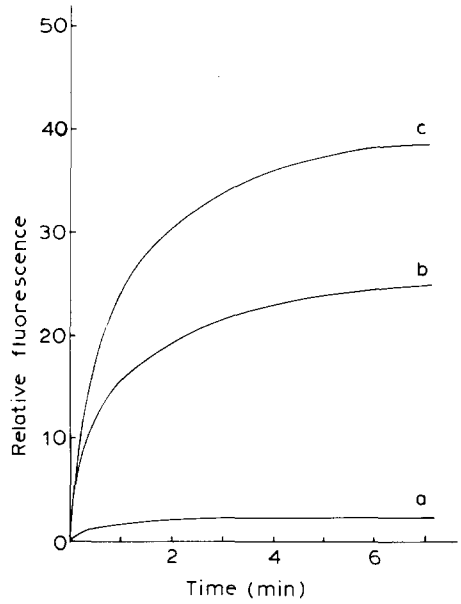

Fig. 1. Effect of *S. cremoris* membrane vesicles on the resonance energy transfer between N-NBD-PE and N-Rh-PE incorporated into bacteriorhodopsin proteoliposomes at pH 4.5. *S. cremoris* membrane vesicles containing 50 nmol phospholipid were added to bacteriorhodopsin proteoliposomes containing 50 nmol phospholipid supplemented with (a) egg PC/N-NBD-PE/N-Rh-Pe (99/0.5/0.5, mol/mol/mol); (b) egg PC/cardiolipin/N-NBD-PE/N-Rh-PE (49..5/49.5/0.5/0.5, mol/mol/mol/mol) or (c) cardiolipin/N-NBD-PE/N-Rh-PE (99/0.5/0.5 mol/mol/mol) in 2 ml 10 mM potassium phosphate buffer (pH 4.5) supplemented with 10 mM sodium acetate and 100 mM NaCl at 25°C.

transfer efficiency, monitored as an increase of the NBD fluorescence (Fig. 1, trace c). When instead of the negatively charged cardiolipin, the neutral phosphatidylcholine was used to reconstitute bacteriorhodopsin, only a low level of interaction could be observed (Fig. 1, trace a). With a mixture of cardiolipin and phosphatidylcholine an intermediate level of NBD fluorescence was observed (Fig. 1, trace b). Dilution of the fluorescent probes originally incorporated into the bacteriorhodopsin proteoliposomes can either occur through transfer of individual phospholipids or by fusion of both membranes. In order to discriminate between these two possibilities, binding of bacteriorhodopsin proteoliposomes to *S. cremoris* membrane vesicles at low pH was studied. *S. cremoris* membrane vesicles were incubated at pH 4.0 with bacteriorhodopsin proteoliposomes labeled with a trace amount of [^{14}C]egg PC. In a parallel experiment *S. cremoris* membrane vesicles were incubated with liposomes either labeled with [^{14}C]egg PC and N-NBD-PE or with ^{14}C cholesteryloleate and N-NBD-PE. After centrifugation the amounts of the different probes (bacteriorhodopsin, [^{14}C]egg PC, [^{14}C]cholesteryloleate and N-NBD-PE) in the supernatant were determined. In both experiments all the different probes disappeared from the supernatant to similar extents (Table I). In the absence of *S. cremoris* membrane

TABLE I

DISAPPEARANCE OF LABELED PHOSPHOLIPIDS AND BACTERIORHODOPSIN FROM THE SUPERNATANT AFTER CENTRIFUGATION OF *S. CREMORIS* MEMBRANE VESICLES FUSED WITH LIPOSOMES OR BACTERIORHODOPSIN PROTEOLIPOSOMES.

Bacteriorhodopsin proteoliposomes (phospholipid/bacteriorhodopsin molar ratio, 160:1) composed of the phospholipids DOPC or DOPC/Cardiolipin (1/1) and a small amount of [^{14}C] egg PC or liposomes composed of DOPC or DOPC/cardiolipin containing 0.5 mol % N-NBD-PE and a small amount of [^{14}C] egg PC or [^{14}C]cholesteryloleate, were fused with *S. cremoris* membrane vesicles as described in Materials and Methods. Aliquots of the suspension were taken for the determination of the total amount of radioactivity, NBD fluorescence and absorption at 568 nm. After centrifugation of the suspension for 5 min, aliquots were taken from the supernatant and the measurements were repeated. The results are expressed as percentage of the marker pelleted by the centrifugation step.

Liposome composition	Percentage of the probes present in pelleted membranes			
	[^{14}C]egg PC	[^{14}C]cholesteryl-oleate	bacterio-rhodopsin	N-NBD-PE
DOPC	9.1	–	–	8.8
	–	11.6	–	6.8
DOPC/cardiolipin	93.3	–	–	95.7
	–	96.2	–	94.0
DOPC/cardiolipin/bacteriorhodopsin	75.8	–	77.9	–

vesicles no pelleting of the bacteriorhodopsin proteoliposomes or liposomes was observed during the short period of centrifugation. These results indicate that association of the liposomes to *S. cremoris* membrane vesicles has occurred. Although this binding experiment cannot discriminate between aggregation, or membrane fusion, the similar extents of binding of the structurally different probes rules out exchange of individual phospholipids. Since the interaction observed with the resonance energy transfer assay cannot be explained by simple aggregation (see Ref. 13), it must be concluded that fusion between both membranes has taken place.

S. cremoris membrane vesicles have a much higher buoyant density than the bacteriorhodopsin proteoliposomes. Therefore, fusion between both membranes should result in a decrease of the buoyant density of *S. cremoris* membrane vesicles. A homogeneous purple pellet was obtained after centrifugation of a suspension of the bacterial membranes mixed at pH 4.0 with bacteriorhodopsin proteoliposomes reconstituted with cardiolipin. This purple pellet was analyzed by centrifugation over a sucrose density gradient in the presence of 500 mM NaCl to avoid aggregation. A fraction was observed with a density intermediate those of *S. cremoris* membrane vesicles and bacteriorhodopsin proteoliposomes (Fig. 2A). This fraction contained bacteriorhodopsin as shown by its visible absorption spectrum (Fig. 2B). Free bacteriorhodopsin proteoliposomes were removed from the fused membranes by centrifugation prior to sucrose density gradient centrifugation. Fusion was also observed between *S. cremoris* membrane vesicles and bacteriorhodopsin proteoliposomes prepared with the neutral phospolipid phosphatidylcholine (Fig. 2A). This is probably due to the presence of a small amount of negatively charged phospholipid from the purple membranes [30].

Theoretically complete fusion of equal quantities of labeled and non-labeled membranes will result in a 50% increase of the relative NBD-fluorescence. At the lowest pH tested (pH 4.0), mixing of *S. cremoris* membrane vesicles and bacteriorhodopsin proteoliposomes resulted in a 42% increase in NBD fluorescence-yield, indicating that efficient fusion has taken place (Fig. 3A). This yield increased with an increasing ratio of *S.*

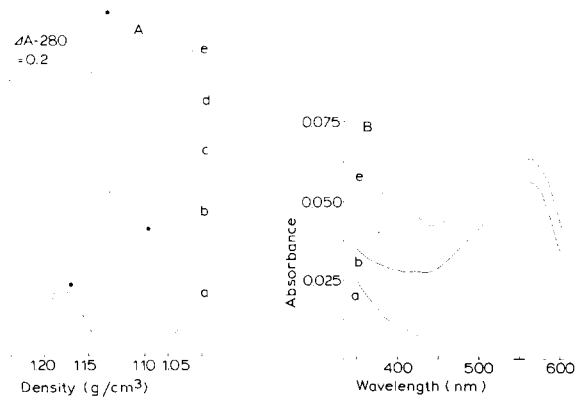

Fig. 2. Analysis of the fusion of *S. cremoris* membrane vesicles with bacteriorhodopsin proteoliposomes by sucrose density gradient centrifugation. (A) Absorbance at 280 nm of the sucrose gradients. (B) Absorption spectra of the peakfractions. (A) *S. cremoris* membrane vesicles (1.8 mg protein) were mixed with bacteriorhodopsin proteoliposomes (phospholipid/bacteriorhodopsin molar ratio, 160:1; 0.8 mg protein) in 2 ml 25 mM potassium phosphate buffer (pH 5.0) supplemented with 25 mM sodium acetate and incubated for 20 min at 4°C. The membranes were washed twice in 50 mM potassium phosphate buffer (pH 7.0) and the resuspended pellet was mixed with 42% (w/v) sucrose in 50 mM potassium phosphate buffer (pH 8.0) containing 1 mM EDTA to a final sucrose concentration of 7% (w/v). (a) *S. cremoris* membrane vesicles only; (b) bacteriorhodopsin proteoliposomes reconstituted with egg PC/cardiolipin (1/1); (c) *S. cremoris* membrane vesicles fused with bacteriorhodopsin proteoliposomes containing egg PC and instead of egg/PC in (d) egg PC/cardiolipin (1/1) and in (e) cardiolipin. (B) Absorption spectra of the peakfractions indicated with an asterisk in (A).

cremoris membrane vesicles to bacteriorhodopsin proteoliposomes (Fig. 3B). The extent of fusion also increased with decreasing pH and increasing amount of cardiolipin, present in the bacteriorhodopsin proteoliposomes (Fig. 3A). In the absence of the negatively charged phospholipid cardiolipin, hardly any fusion was observed (Fig. 3A).

Generation of a protonmotive force in the fused membranes

Bacteriorhodopsin reconstituted into liposomes by the use of the sonication procedure translocates proton into the proteoliposomes [13,27]. The orientation of bacteriorhodopsin is opposite to that in whole cells of *Halobacterium halobium.* Consequently bacteriorhodopsin will generate a reversed protonmotive force (interior positive and acid upon illumination.

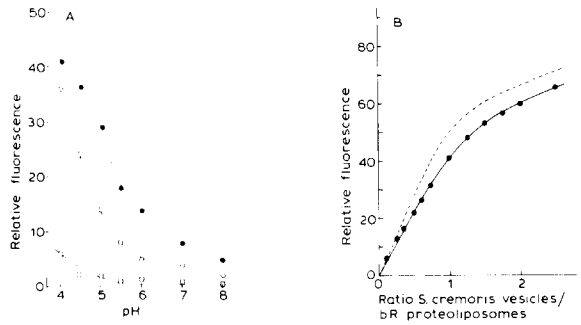

Fig. 3. Effect of the phospholipid weight ratio of *S. cremoris* membrane vesicles and bacteriorhodopsin (bR) proteoliposomes (B) and the fusion pH (A) on the energy-transfer efficiency between N-NBD-PE and N-Rh-PE incorporated into bacteriorhodopsin proteoliposomes. (A) 50 nmol *S. cremoris* membrane vesicles were added to 50 nmol bacteriorhodopsin proteoliposomes (phospholipid/bacteriorhodopsin molar ratio 160:1) reconstituted with egg PC/N-NBD-PE/N-Rh-PE (99/0.5/0.5). (□), egg PC/cardiolipin/N-NBD-PE/N-Rh-PE (49.5/49.5/0.5/0.5) (○) or cardiolipin/N-NBD-PE/N-Rh-PE (99/0.5/0.5) (●) at the indicated pH. The maximal relative 7-nitro-2,1,3-benzoxadiazol-4-yl fluorescence was determined from the steady-state levels of 7-nitro-2,1,3-benzoxadiazol-4-yl fluorescence with TX-100. (B) *S. cremoris* membrane vesicles, containing 0 to 125 nmol phospholipid were added to bacteriorhodopsin proteoliposomes (phospholipid/bacteriorhodopsin molar ratio, 160:1) containing 50 nmol phospholipid supplemented with cardiolipin/N-NBD-PE/N-Rh-PE (99/0.5/0.5) at pH 4.0. The theoretical derived curve is indicated by the broken line (— — —).

Measurements of the accumulation of the lipophilic anion tetraphenylboron (TPB^-), performed with a tetraphenylphosphonium (TPP^+) selective electrode [21] which also responds to TPB^- [20], indicated that at saturating light intensities and in the absence of a ΔpH, a $\Delta\psi$ of about +75 to +80 mV was generated in these proteoliposomes (not shown). Under similar conditions no uptake of TPP^+ was observed indicating that the majority of the bacteriorhodopsin proteoliposomes generated a reversed protonmotive force.

In *S. cremoris* membrane vesicles fused with bacteriorhodopsin proteoliposomes a $\Delta\psi$ of +45 to +50 mV could be generated in the light as estimated from the accumulation of TPB^-. Similar values were obtained with the $\Delta\psi$ probe SCN^-. The orientation of bacteriorhodopsin did not change significantly as a result of the fusion procedure, since hardly any TPP^+ uptake was detectable in the fused membranes upon illumination. With the flow-dialysis technique a ΔpH of about +42 mV could be estimated from the uptake of methylamine by the fused membranes upon illumination. From $\Delta\psi$ or ΔpH measurements alone it is not possible to determine whether bacteriorhodopsin is actually functionally incorporated in the *S. cremoris* membrane vesicles.

In order to establish whether bacteriorhodopsin indeed generated a protonmotive force in the fused membranes, bacteriorhodopsin proteoliposomes were fused with *S. cremoris* membrane vesicles pretreated with different concentrations of dicyclohexylcarbodiimide (DCCD) and the effect of this DCCD treatment on the proton permeability of the fused membranes was studied. Treatment of bacterial membrane vesicles with low concentrations of DCCD has a stimulating effect on the magnitude of an artificially imposed $\Delta\psi$ (see Ref. 31 and data not shown). This is presumably due to blockage of passive proton leakage via the F_0 component of the membrane bound F_1F_0 ATPase that has become partially dissociated during the isolation of membrane vesicles.

The relative passive proton permeability of bacteriorhodopsin proteoliposomes and the fused membranes can be determined from the external pH changes when illumination is turned off. Eisenbach et al. [32] showed that the decay of the alkalinisation observed in the dark is the sum of two exponentials, the first phase being faster than the second. The rapid phase represents a $\Delta\psi$ dependent reaction [33], while the slow phase represents net proton transport across the membrane [32]. Since this slow phase reflects back diffusion of protons under influence of the protonmotive force, it will be limited by the flux of accompanying ions as well as by the proton permeability. Therefore, it can be used to determine the relative proton permeability of bacteriorhodopsin proteoliposomes and the fused membranes. When bacteriorhodopsin proteoliposomes are fused with *S. cremoris* membrane vesicles a decrease of the half-time of proton back diffusion could be observed (Fig. 4A), indicating an increase in the relative proton permeability with respect to the liposomal membrane. This decrease in half-time was less pronounced when *S. cremoris* membrane vesicles were pretreated with DCCD (Fig. 4). It should be emphasized that DCCD treatment of

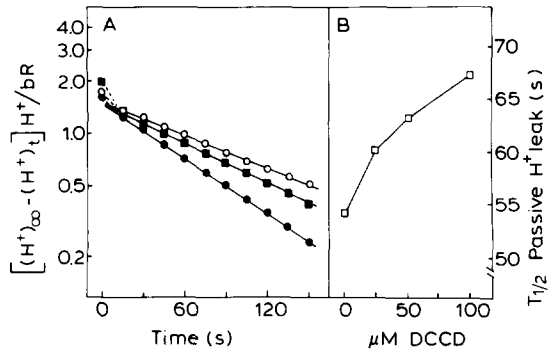

Fig. 4. Effect of DCCD pretreatment of *S. cremoris* membrane vesicles on the passive back diffusion of protons in *S. cremoris* membrane vesicles fused with bacteriorhodopsin (bR) proteoliposomes. (A) Semilogarithmic traces of proton back diffusion (○), bacteriorhodopsin proteoliposomes only, (●) *S. cremoris* membrane vesicles fused with bacteriorhodopsin proteoliposomes and (■) *S. cremoris* membrane vesicles pretreated with 100 μM DCCD (40 nmol DCCD/mg protein) fused with bacteriorhodopsin proteoliposomes. *S. cremoris* membrane vesicles were washed twice with 0.15 M KCl (pH 6.0) prior to fusion. (B) Half-time of the passive back diffusion protons as a function of the concentration of DCCD, with which *S. cremoris* membrane vesicles were treated prior to fusion.

the *S. cremoris* membrane vesicles had no significant effect on the fusion efficiency as measured with the resonance energy transfer assay (not shown).

Calcium uptake by the fused membranes

Since the protonmotive force generated by bacteriorhodopsin in the fused membranes upon illumination is reversed with respect to the direction of the protonmotive force in whole cells of *S. cremoris,* it is in principle only possible to study the uptake of solutes normally extruded by whole cells. A large number of bacteria extrudes calcium via a Ca/H^+ antiport mechanism (for a review, see Ref. 34). Membrane vesicles of *S. cremoris* accumulate calcium when an artificial ΔpH (interior acid) is imposed by a nigericin catalyzed potassium diffusion gradient (Driessen, A.J.M. and Konings, W.N., unpublished results). It is therefore of interest to test whether calcium is accumulated by *S. cremoris* membrane vesicles fused with bacteriorhodopsin proteoliposomes upon illumination. When *S. cremoris* membrane vesicles fused with bacteriorhodopsin proteoliposomes at pH 5.0, were illuminated (1 $kJ \cdot m^{-2} \cdot s^{-1}$), calcium uptake could be observed (Fig. 5). Ca^{2+} uptake was stimulated by valinomycin (5 μM) and inhibited by nigericin (0.5 μM). In the presence of the uncouplers CCCP (10 μM) or 5 chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanilide (S-13) (5 μM), or in the presence of both valinomycin and nigericin, only binding of the calcium to the vesicles was observed. Bleaching of bacteriorhodopsin in the presence of hydroxylamine and light also inhibited calcium uptake. This effect was only observed with freshly prepared hydroxylamine solutions. Control experiments with bacteriorhodopsin proteolipo-

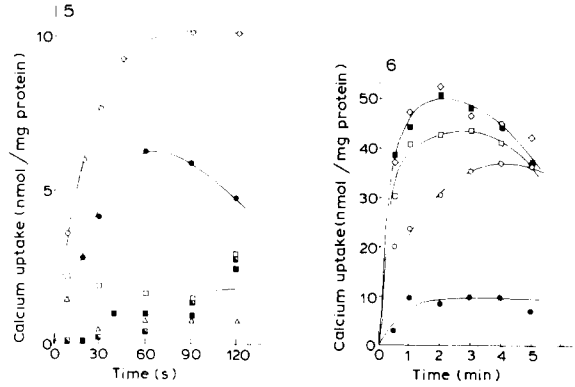

Fig. 5. Calcium uptake by *S. cremoris* membrane vesicles fused with bacteriorhodopsin proteoliposomes. *S. cremoris* membrane vesicles were fused with bacteriorhodopsin proteoliposomes, containing cardiolipin, at pH 5.0, as described in Materials and Methods. Ca^{2+} uptake was studied with the filtration technique in 50 mM potassium phosphate (pH 7.0) supplemented with 10 mM $MgSO_4$ at a light intensity of 1 $kJ \cdot m^{-2} \cdot s^{-1}$. The incubation mixture contained 0.5 mM $^{45}Ca^{2+}$ and 1 mg/ml membrane protein. The conditions were (●) no additions; (□) 0.5 μM nigericin; (○) 5 μM valinomycin; (△) 10 μM CCCP or (■) in the dark. (◪) Ca^{2+} uptake in *S. cremoris* membrane vesicles fused with proteoliposomes, in which bacteriorhodopsin, bleached with NH_2OH, was incorporated.

Fig. 6. Effect of the light intensity on calcium uptake by *S. cremoris* membrane vesicles fused with bacteriorhodopsin proteoliposomes. *S. cremoris* membrane vesicles were fused with bacteriorhodopsin proteoliposomes, containing Cl, at pH 5.0 as described in Materials and Methods. Ca^{2+} uptake was performed by the filtration technique in 50 mM potassium phosphate (pH 8.0) supplemented with 2 mM $MgSO_4$ at various light intensities. The incubation mixture contained 0.5 mM $^{45}Ca^{2+}$ and 1.5 mg/ml membrane protein. Vesicles were preincubated with 5 μM valinomycin. Light intensities: (●) dark; (○) 30%; (□) 45%; (■) 60% or (◇) 80% of the maximal intensity (1.5 $kJ \cdot m^{-2} \cdot s^{-1}$).

somes or *S. cremoris* membrane vesicles alone did not show any significant uptake of calcium upon illumination.

Uptake of calcium increased with increasing light intensities (Fig. 6) up to saturation. Particularly at high light intensities, the Ca^{2+} uptake reached its maximum level after 1–2 min, after which the Ca^{2+} leaked out.

Discussion

In this paper we have shown that the light-driven proton pump bacteriorhodopsin can be functionally incorporated in membrane vesicles of *Streptococcus cremoris* by fusion of these vesicles with bacteriorhodopsin proteoliposomes. Fusion between *S. cremoris* membrane vesicles and bacteriorhodopsin proteoliposomes, containing a high amount of cardiolipin, occurred when both preparations were mixed at low pH (less than 6.0). Recently, several reports appeared about low pH stimulated fusion processes showing a similar requirement for negatively charged phospholipids [12,35]. Fusion of liposomes with *Bacillus subtilis* or *Escherichia coli* membrane vesicles [12], *Rhodopseudomonas sphaeroides* chromatophores [35,36], or mitochondrial inner membranes [37,38] is also stimulated by incubation at low pH.

Fusion of *S. cremoris* membrane vesicles and bacteriorhodopsin proteoliposomes was demonstrated by (i) dilution of the non-exchangeable fluorescent resonance energy transfer phospholipid probes, originally incorporated in the bacteriorhodopsin proteoliposomes only, (ii) formation of interaction products with an intermediate buoyant density and (iii) by light dependent calcium transport. Incorporation of bacteriorhodopsin into the liposomes did not negatively affect the fusion efficiency. Fusion between *S. cremoris* membrane vesicles and bacteriorhodopsin proteoliposomes was very similar to fusion of *B. subtilis* membrane vesicles and liposomes, with respect to the pH dependency and the specific requirement for negatively charged phospholipids in the liposomal bilayer [12]. We therefore concluded that *S. cremoris* membrane vesicles and *B. subtilis* membrane vesicles have similar fusogenic properties.

Bacteriorhodopsin proteoliposomes prepared by the sonication procedure pump protons into the vesicles upon illumination. Fusion with the *S. cremoris* membrane vesicles did not change the direction of the proton pump. Consequently a $\Delta\psi$ interior positive and a ΔpH interior acidic was generated upon illumination. The $\Delta\psi$ and ΔpH values were relatively low compared to the values normally found in bacteriorhodopsin proteoliposomes. Recently, Casadio and Sorgato [39] reported ΔpH generation by bacteriorhodopsin in submitochondrial particles fused with bacteriorhodopsin proteoliposomes by means of a freezing and thawing plus sonication technique. Although they were able to show light dependent ATP synthesis in the fused membranes, they also observed a decline in the ΔpH generated by bacteriorhodopsin in the fused membranes compared to the ΔpH generated in the bacteriorhodopsin proteoliposomes (the ΔpH dropped from 150 mV to about 55 mV). This lower protonmotive force in the fused membranes is due to a higher ion permeability of the fused membranes compared to bacteriorhodopsin proteoliposomes (see Fig. 4). Fused membranes are relatively heterogeneous with respect to size and bacteriorhodopsin molecule distribution. This heterogeneity will be the subject of a more detailed study in which water-soluble fluorescent pH indicators will be used to measure more accurately the pH in the fused membranes only.

Unequivocal evidence for functional incorporation of bacteriorhodopsin in the *S. cremoris* membrane vesicles cannot be supplied by protonmotive force measurements alone. Therefore the effect of DCCD on the passive proton permeability of the fused membranes was studied. Treatment of *S. cremoris* membrane vesicles with DCCD prior to fusion resulted in fused membranes with a slower proton back diffusion than fused membranes prepared from *S. cremoris* membrane vesicles which were not treated with DCCD. Since DCCD had no significant effect on the fusion efficiency, these results can only be explained when bacteriorhodopsin indeed operates functionally in the fused membranes. More evidence in favour of a functional incorporation of bacteriorhodopsin in the fused membranes is obtained by the demonstration of light-driven uptake of calcium. Since bacteriorhodopsin has an inside-out orientation in the fused membranes, solute uptake studies are

limited to extrusion systems, such as those for calcium and sodium. We examined calcium uptake here. Uptake of calcium increased with increasing light intensity. In the dark, or when bleached bacteriorhodopsin was used, only binding of calcium was observed. Since neither bacteriorhodopsin proteoliposomes nor *S. cremoris* membrane vesicles showed any significant light-dependent calcium uptake, these results have to be explained as uptake of calcium by the fused membranes via a calcium transport system present in the *S. cremoris* membranes. Uptake of calcium was stimulated by valinomycin and inhibited by nigericin and uncouplers. These results indicate that Ca^{2+} is transported by a Ca^{2+}/H^{+} antiport mechanism. More evidence for such a Ca^{2+}/H^{+} antiport system in *S. cremoris* membrane vesicles has recently been obtained and will be published elsewhere.

With the system described in this report (with bacteriorhodopsin pumping protons into the fused membranes) the uptake of solutes normally extruded by whole cells can be studied. Experiments have been started to incorporate bacteriorhodopsin in a right-side out orientation in the fused membranes by the use of right-side out oriented bacteriorhodopsin proteoliposomes [14,28]. Such a system hopefully will result in a modelsystem to study H^{+}/amino acid symport in lactic acid streptococci and other anaerobic fermentative bacteria lacking a functional electron-transfer chain.

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