

Light-Driven Amino Acid Uptake in *Streptococcus cremoris* or *Clostridium acetobutylicum* Membrane Vesicles Fused with Liposomes Containing Bacterial Reaction Centers

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Reaction centers of the phototrophic bacterium *Rhodospseudomonas palustris* were introduced as proton motive force-generating systems in membrane vesicles of two anaerobic bacteria. Liposomes containing reaction center-light-harvesting complex I pigment protein complexes were fused with membrane vesicles of *Streptococcus cremoris* or *Clostridium acetobutylicum* by freeze-thawing and sonication. Illumination of these fused membranes resulted in the generation of a proton motive force of approximately -110 mV. The magnitude of the proton motive force in these membranes could be varied by changing the light intensity. As a result of this proton motive force, amino acid transport into the fused membranes could be observed. The initial rate of leucine transport by membrane vesicles of *S. cremoris* increased exponentially with the proton motive force. An H^+ /leucine stoichiometry of 0.8 was determined from the steady-state level of leucine accumulation and the proton motive force, and this stoichiometry was found to be independent of the magnitude of the proton motive force. These results indicate that the introduction of bacterial reaction centers in membrane vesicles by the fusion procedure yields very attractive model systems for the study of proton motive force-consuming processes in membrane vesicles of (strict) anaerobic bacteria.

The incorporation of primary proton pumps in biological membranes has opened attractive possibilities for studies of proton motive force (Δp)-dependent processes in isolated membrane vesicles of bacterial (4, 5) and eucaryotic (18) origin (for a review, see reference 7). Fused membranes obtained from liposomes containing a Δp -generating system and membrane vesicles of fermentative bacteria lacking an accessible proton pump have been shown to be excellent model systems for studies on the role of the Δp in solute transport (3-10). Cytochrome *c* oxidase (4, 5) and bacteriorhodopsin (6, 10) have been used extensively as Δp -generating systems in these fused membranes. Both proton pumps are able to maintain a large Δp for a considerable length of time, which is a large advantage over the use of (usually transient) artificial Δp gradients. A light-driven proton pump such as bacteriorhodopsin has the advantage over cytochrome *c* oxidase in that variations in Δp levels can be achieved easily by varying the light intensity. It is, however, difficult to control the orientation of bacteriorhodopsin after fusion. Fusion of bacteriorhodopsin liposomes with membrane vesicles usually leads to a reversed orientation of the bacteriorhodopsin molecules, so that protons are pumped into the fused membranes upon illumination (6). Bacteriorhodopsin is therefore only suitable for studies on solute extrusion systems.

The Δp -generating system cytochrome *c* oxidase in fused membranes always generates a Δp of the in vivo polarity (inside negative and alkaline). Only those cytochrome *c* oxidase molecules that interact with externally added reduced cytochrome *c* participate in proton pumping. This system is therefore very useful for the study of solute uptake processes. Variations in Δp levels, however, always make it necessary to change the concentrations of the used redox mediators or add uncouplers or protonophores.

Recently we showed that reaction centers (RCs) of phototrophic bacteria can be successfully and functionally reconstituted into liposomes (13, 14; D. Molenaar, W. Crielgaard, and K. J. Hellingwerf, *Biochemistry*, in press). At pH 7.0, in the presence of cytochrome *c*, ubiquinone-0, and ascorbic acid, a light-induced Δp of approximately -170 mV can be generated in these liposomes (Molenaar et al., in press). In this paper, we show that these RCs can also be functionally incorporated into bacterial membranes by fusing RC-liposomes with bacterial membrane vesicles.

MATERIALS AND METHODS

Materials. Radioactive amino acids were purchased from the Radiochemical Centre, Amersham (Buckinghamshire, U.K.). Octyl- β -D-glucopyranoside and horse heart cytochrome *c* were obtained from Sigma Chemical Co. (St. Louis, Mo.). All other materials were reagent grade and obtained from commercial sources.

Growth of *Rhodospseudomonas palustris* and isolation of RCLH₁ complexes. *R. palustris* NCIB 8828 was grown anaerobically under low light intensity on the medium described by Sistro (21) in a 20-liter flask at 30°C as described previously (Molenaar et al., in press). RC-light-harvesting complex I pigment proteins (RCLH₁ complexes; the bacterial RC with the antenna protein B875 still attached) were isolated from chromatophores of these cells by *n*-octyl- β -D-glucopyranoside-deoxycholate extraction, followed by sucrose density centrifugation (Molenaar et al., in press). RCLH₁ complexes were collected from the gradients and stored in small portions in liquid nitrogen.

Growth of bacteria and isolation of membrane vesicles. *Streptococcus cremoris* Wg2 (*pri*) and *Clostridium acetobutylicum* ATCC 824 were grown on MRS broth (2) at a controlled pH of 6.4 in a 5-liter fermentor. With *C. acetobutylicum*, special precautions were taken to prevent leakage of oxygen during growth. Membrane vesicles of *S.*

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cremoris (19) and of *C. acetobutylicum* (11) were prepared as described previously. Membrane vesicles were suspended in 50 mM potassium phosphate, pH 7.0, supplemented with 10 mM MgSO₄ at a concentration of 10 to 15 mg of protein per ml and stored in liquid nitrogen.

Reconstitution of RCLH₁ complexes into liposomes and fusion of RC-liposomes with bacterial membrane vesicles. RC complexes were incorporated into liposomes prepared from acetone- and ether-washed *Escherichia coli* phospholipid (4) by a combination of dialysis and sonication (at a ratio of 1.4 nmol of RC per mg of lipid) as described previously (Molenaar et al., in press). Membrane vesicles and RCLH₁-liposomes were mixed (1 mg of membrane vesicle protein per 10 mg of liposome lipid) and rapidly frozen in liquid nitrogen. The mixture was then slowly thawed at room temperature and sonicated two times for 3 s each at 0°C under a constant stream of nitrogen with an MSE probe-type sonicator (MSE Scientific Instruments, West Sussex, U.K.). Fused membranes were collected by centrifugation for 1 h at 55,000 rpm (maximum, 280,000 × *g*) in a Beckman type 75 Ti rotor at 4°C and suspended to a protein concentration of approximately 10 mg/ml in 20 mM K-HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid adjusted to pH 7.0 with KOH)–50 mM KCl.

Uptake assays and electrical potential measurements. $\Delta\psi$ -driven amino acid uptake and the electrical potential across the membrane ($\Delta\psi$) were measured simultaneously in a temperature-controlled vessel at 20°C. Where indicated, anaerobiosis was achieved by a constant flow of water-saturated oxygen-free nitrogen over the surface of the incubation mixture. Actinic light supplied by a projector lamp (24 V, 150 W) was transferred to the vessel via a light guide. The light intensity was varied by changing the voltage over the projector lamp. The incubation mixture contained 20 mM K-HEPES (pH 7.0), 50 mM KCl, 0.5 mM MgCl₂, 0.5 mM ascorbic acid, 20 μ M cytochrome *c*, 400 μ M ubiquinone-0, and 2 μ M tetraphenylphosphonium (TPP⁺) in a total volume of 2 ml. L-[¹⁴C]leucine (12.4 TBq/mol), L-[¹⁴C]serine (6.6 TBq/mol), and L-[¹⁴C]lysine (12.4 TBq/mol) were added to final concentrations of 4.5, 9, and 4.5 μ M, respectively. To prevent the build-up of a pH gradient across the membrane (Δ pH), nigericin was included in all experiments at a concentration of 20 nM.

The distribution of TPP⁺ over the liposomal membrane was determined with a TPP⁺-selective electrode (constructed by the method of Shinbo et al. [20]). The electrochemical potential was calculated from this distribution with the Nernst equation. A correction for concentration-dependent binding of TPP⁺ to the liposomal membrane was made by the model of Lolkema et al. (15).

For uptake studies, samples (50 μ l) were taken from the incubation mixture, diluted in 2 ml of 100 mM LiCl, and filtered over cellulose nitrate filters (45- μ m pore size). The filters were washed once with 2 ml of 100 mM LiCl, dried, and transferred to scintillation vials. The radioactivity on the filters was measured after the addition of scintillation fluid with a liquid scintillation counter (Packard Tri-Carb 460 CD; Packard Instruments Corp.).

Analytical methods. Protein was determined by the method of Lowry et al. (16). RC concentrations were calculated from the absorption difference at 880 nm between dithionite-reduced and ferricyanide-oxidized RCs (12) at an absorption coefficient of 113 mM⁻¹ cm⁻¹ (1).

The internal volume of the fused membranes was determined by the calceine quenching method (17) and was found to be 5.5 μ l/mg of protein for fused membranes of *S.*

cremoris membrane vesicles and 4.6 μ l/mg of protein for fused membranes of *C. acetobutylicum*.

Light intensity was measured with a YSI-Kettering no. 65 radiometer.

RESULTS

$\Delta\psi$ generation in *S. cremoris* membrane vesicles fused with RC-liposomes. In the light, cyclic electron transfer occurs in RC-proteoliposomes when cytochrome *c* and ubiquinone-0 are present as redox mediators. Electron transfer leads to the generation of a $\Delta\psi$ across the liposomal membrane (Molenaar et al., in press). This type of electron transport and $\Delta\psi$ generation only proceeds at alkaline pH (>8.0), because the oxidation of ubiquinol-0 by cytochrome *c* is pH dependent. The pH range of the light-driven $\Delta\psi$ generation can be extended to lower pHs by adding small amounts of ascorbic acid to the liposomes as an extra electron donor for cytochrome *c*. It is thus possible to maintain a light-induced $\Delta\psi$ of ca. -170 mV for at least 30 min in RC-liposomes at pH 7.0. Under these conditions, RCs could be used for the generation of a $\Delta\psi$ in fused membranes. Therefore, it was tested whether the $\Delta\psi$ -generating capacities of the RCs were affected by freeze-thaw and sonication fusion with *S. cremoris* membrane vesicles. First, it was checked whether the orientation of the RCs in the membrane changed upon fusion. The re-reduction of flash-bleached P₈₈₀ (the special pair bacteriochlorophyll) in the presence and absence of cytochrome *c* was followed spectrophotometrically (14; Molenaar et al., in press). These studies revealed that more than 95% of the RC population in the fused membranes was orientated as in vivo, indicating that fusion had no negative effect (i.e., did not reduce the number of right-side-oriented RCs) on the orientation. Since cytochrome *c* was added to the outside of the fused membranes, illumination should lead to an outward pumping of protons and the generation of a $\Delta\psi$. Under the experimental conditions used, only a $\Delta\psi$ could be generated, which was recorded from the distribution of TPP⁺ between the external bulk phase and the inner compartment of the fused membrane vesicles. Figure 1 shows a typical recording of a TPP⁺ distribution experiment. Addition of fused membranes to a medium containing TPP⁺ led to a drop in the external TPP⁺ concen-

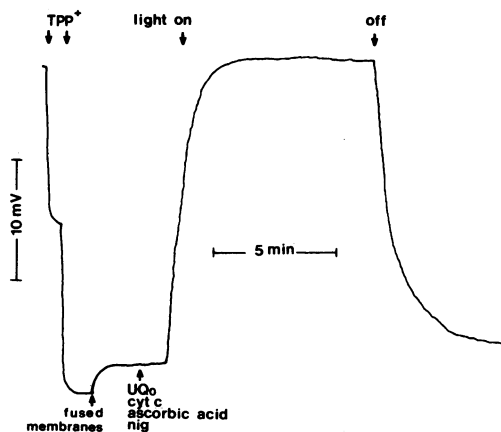


FIG. 1. Effect of illumination on the accumulation of TPP⁺ in *S. cremoris* membrane vesicles fused with RC-liposomes. Experiments were performed in the presence of ubiquinone-0 (UQ₀, 400 μ M), cytochrome *c* (cyt *c*, 20 μ M), ascorbic acid (500 μ M), and nigericin (nig, 20 nM), as described in Materials and Methods.

tration due to dilution and binding of TPP^+ to the membranes. In the dark, addition of ubiquinone-0, cytochrome *c*, and ascorbic acid (to create a redox cycle) and nigericin (to prevent the possible build-up of a ΔpH) had no effect on the distribution of TPP^+ . Illumination of the suspension led to a rapid drop of the external TPP^+ concentration, indicating accumulation of TPP^+ by the fused membranes. In the light, the extent of TPP^+ accumulation remained constant over several minutes, indicating a stable $\Delta\psi$. The $\Delta\psi$ generated in the light in these fused membranes was approximately -110 mV. Turning off the light led to a very rapid efflux of TPP^+ from the liposomes, indicating a rapid decrease of the $\Delta\psi$.

Light-driven leucine transport in the fused membranes. To investigate whether the membranes obtained by fusion of *S. cremoris* membrane vesicles and RC-liposomes were coupled, i.e., whether the Δp generated by the RCs could be used by one of the Δp -consuming processes in the streptococcal membrane, the uptake of leucine in the fused membrane was followed (Fig. 2). In the dark, the uptake of leucine was very slow and the level of uptake did not exceed equilibration. In the light, however, leucine was taken up rapidly. Initial rates of leucine transport of 0.54 nmol/mg of protein per min were observed. A steady-state level of leucine uptake was reached within 4 min after the addition of leucine. When the light was switched off, efflux of the accumulated leucine occurred immediately, and within 4 min the internal leucine concentration equaled the external concentration under dark conditions. Addition of the uncoupler carbonyl-cyanide-*m*-chlorophenylhydrazone (CCCP) also resulted in complete dissipation of the Δp and collapse of the leucine gradient across the fused membrane (Fig. 2).

As was expected, uptake of leucine could not be detected in nonfused liposomes or nonfused (and therefore nonenergized) membrane vesicles (see also reference 4). Uptake of leucine in the fused membranes is therefore strong evidence for functional incorporation of the RCs in the *S. cremoris* membranes.

It should be noted (although the data are not presented) that it is possible to study not only the relatively rapid uptake of leucine in the fused membrane system, but also the much slower process of serine uptake (9). Steady-state levels of serine accumulation were achieved at pH 7.0 after more than

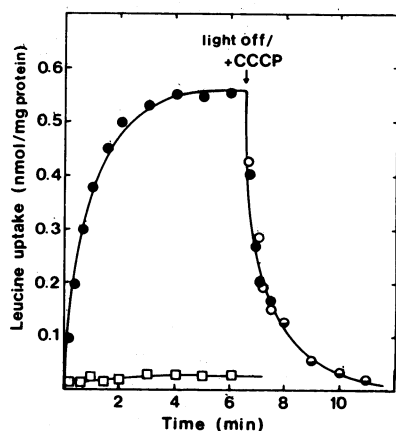


FIG. 2. Uptake of leucine by *S. cremoris* membrane vesicles fused with RC-liposomes. Experiments were performed as described in Materials and Methods at maximum light intensity (9.0 kW/m 2). Uptake was started by adding [^{14}C]leucine (4.5 μM). At the arrow, the light was switched off (●) or CCCP (2 μM) was added (○).

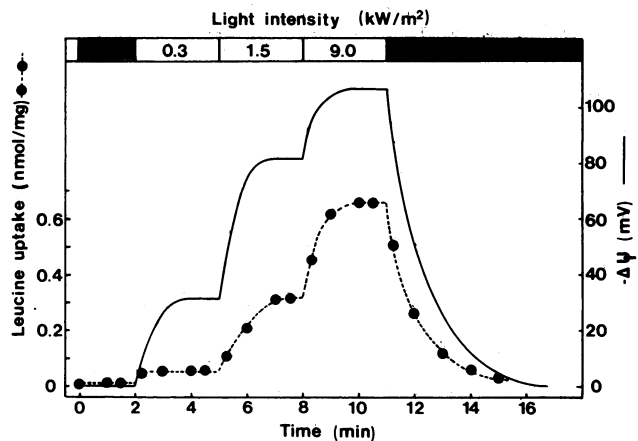


FIG. 3. Electrical potential (—) and uptake of leucine (---) in *S. cremoris* membrane vesicles fused with RC-liposomes at increasing light intensities. Experiments were performed as described in Materials and Methods. The $\Delta\psi$ and the uptake of leucine were measured simultaneously.

15 min of illumination, indicating that a Δp can be maintained in the fused membranes for that long.

Relation between Δp generation and leucine uptake. In RC-liposomes, the Δp generated depends on the light intensity used for energization (Molenaar et al., in press). This appeared also to be the case in the fused membranes. Figure 3 shows a simultaneous recording of both the Δp and the uptake of leucine in the fused membranes. It is clear that at increasing light intensities both the Δp and the level of accumulated leucine increased. This behavior was investigated in more detail. Both Δp and the accumulation level of leucine increased in parallel with increasing light intensities up to 3.4 kW/m 2 (data not shown).

Since both the Δp and the steady-state level of leucine accumulation can be varied in these fused membranes by varying the light intensity, the relationship between Δp and $\Delta\text{p}_{\text{Leu}} [= (2.3RT/F)\log([\text{leucine}]_{\text{in}}/[\text{leucine}]_{\text{out}})]$, where R , T , and F are the gas constant, the absolute temperature, and the Faraday constant, respectively] can easily be studied. The steady-state level of accumulation of leucine depended linearly on the Δp , indicating a constant proton/leucine stoichiometry at different Δp values. An apparent stoichiometry of 0.8 can be calculated from the slope of the linear relationship (Fig. 4). This apparent stoichiometry was independent of the way a certain Δp level was achieved: starting from a Δp of 0 mV and following uptake at a fixed Δp (as in Fig. 2) or increasing the Δp in one experiment and following uptake upon increasing the Δp (as in Fig. 3).

Dependence of initial rate of uptake on Δp . It has been shown that the rate of amino acid uptake in membrane vesicles of *S. cremoris* depends sharply on the magnitude of Δp (3). To investigate this phenomenon in more detail, the initial rate of leucine uptake was measured at different light intensities (i.e., at different Δp levels). The initial rate was calculated from the uptake of leucine 30 s after the addition of leucine. The Δp was determined simultaneously. Figure 5 shows that the initial rate of leucine uptake varied exponentially with the Δp . A logarithmic plot of the data (Fig. 5, inset) yielded a linear relationship.

Amino acid accumulation in membrane vesicles of *C. acetobutylicum* fused with RC-liposomes. To investigate whether RCs could be used as an efficient Δp -generating system in membranes of strict anaerobic bacteria, membrane vesicles

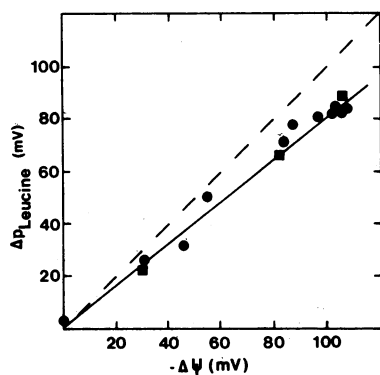


FIG. 4. Relationship between the accumulation level of leucine (Δp_{Leu}) and $\Delta\psi$ in *S. cremoris* membrane vesicles fused with RC-liposomes. Data were collected as described in the legends to Fig. 2 (●) and Fig. 3 (■).

of the strict anaerobic bacterium *C. acetobutylicum* were fused with RC-liposomes and the uptake of amino acids was studied in these fused membranes under anaerobic conditions. Upon illumination, uptake of the amino acids leucine and lysine into these fused membranes occurred (Fig. 6). Steady-state levels of accumulation of 15-fold for lysine and 10-fold for leucine were reached within 10 min. When the light was switched off, both amino acids leaked out of the fused membranes.

DISCUSSION

This study shows that bacterial RCs can be functionally incorporated in membrane vesicles of fermentative bacteria. Upon fusion of the membranes, there was no reorientation of the RC complexes, which were incorporated in the liposomal membranes almost completely in an *in vivo* orientation (Molenaar et al., *in press*). Consequently, also in the fused

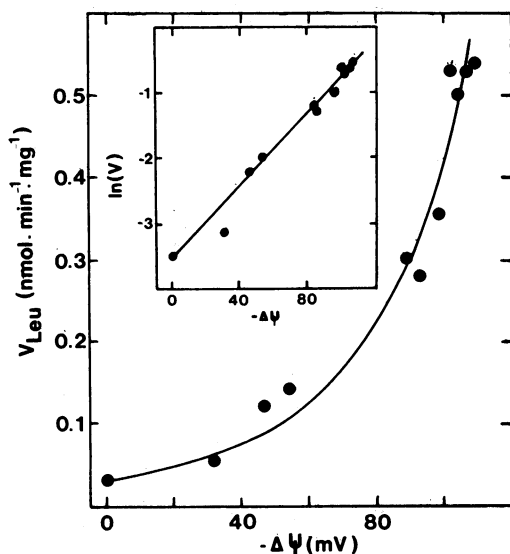


FIG. 5. Relationship between the initial rate of leucine uptake (V_{Leu}) and $\Delta\psi$ in *S. cremoris* membrane vesicles fused with RC-liposomes. Data were calculated from the uptake 30 s after the addition of leucine from experiments analogous to those described in the legend to Fig. 2. The inset shows a logarithmic plot of the data.

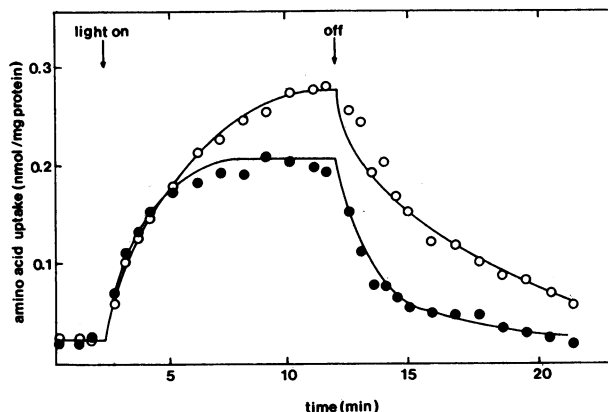


FIG. 6. Uptake of leucine (●) and lysine (○) in *C. acetobutylicum* membrane vesicles fused with RC-liposomes. Uptake was started by switching on the light (9.0 kW/m^2) and terminated by switching the light off. [^{14}C]leucine and [^{14}C]lysine were added to final concentrations of $4.5 \mu\text{M}$. Experiments were performed as described in Materials and Methods.

membranes, almost all RCs were incorporated with the cytochrome *c*-binding site exposed to the outer surface. Illumination of the fused membranes of RC-liposomes with membrane vesicles resulted in the generation of a Δp of ca. -110 mV across the vesicle membrane, as indicated by TPP^+ uptake (Fig. 1). This Δp was considerably lower than the Δp that can be generated in RC-liposomes (-170 mV) (Molenaar et al., *in press*). This may be due to a decline in the RC activity or to higher proton or ion permeability of the fused membranes. However, the Δp generated was still in a useful range.

The light-induced Δp could drive the uptake of several amino acids into the fused membranes (Fig. 2 and 6). An attractive feature of this system is that the Δp in these fused membranes can be varied simply by changing the light intensity, which makes it possible to study the relationship between Δp and the rates and steady-state levels of amino acid transport.

A linear relationship was found between the steady-state level of accumulation of leucine and the Δp . The slope of this linear relationship depended on the number of protons symported with one molecule of leucine by the leucine carrier and on the rate of non-carrier-mediated efflux (passive diffusion) of leucine across the lipid membrane (8). A $\Delta p/\Delta p_{\text{Leu}}$ ratio of 0.8 was observed over the total range of Δp 's studied (Fig. 4). Considering the relatively high rate of passive diffusion of leucine across the membrane (8), this experimentally determined ratio of 0.8 indicates a most likely mechanistic proton-to-leucine stoichiometry of 1. The same stoichiometry was estimated for membranes of *S. cremoris* membrane vesicles fused with proteoliposomes containing beef heart cytochrome *c* oxidase (8, 9).

In this study an exponential relationship was found between the initial rate of uptake of leucine and the Δp (Fig. 5). Since the K , (the apparent K_m for transport) was found to be independent of Δp (3), it is to be expected that V_{max} also increases exponentially with the Δp . The reason for this phenomenon is not yet known.

A potentially attractive feature of the fused membrane model system used in this study is that RCs can be used as proton pumps under strict anaerobic conditions, which allows studies of Δp -dependent processes in membranes of strict anaerobic bacteria. This is demonstrated by the uptake

of leucine and lysine by membrane vesicles of *C. acetobutylicum* fused with RC-liposomes (Fig. 6).

By using RCs as a Δp -generating system, the attractive features of both bacteriorhodopsin and cytochrome *c* oxidase are combined. As for bacteriorhodopsin, it is possible to control exactly the start and stop of proton-pumping activity by switching the light on and off and the rate of proton pumping by varying the light intensity. The Δp and any desired magnitude up to the maximum level can thus be generated and controlled at any time. As for cytochrome *c* oxidase, the direction of proton pumping is always from the inner bulk phase to the outer bulk phase, since cytochrome *c* is added at the outside and interacts only with properly oriented RCs.

The major disadvantage of this pump is its narrow useful pH range. Because light-driven cyclic electron flow is strictly dependent on the pH-sensitive chemical reaction between cytochrome *c* and ubiquinol-0, Δp generation by the RCs is only possible at relatively high pHs (≥ 8.0). The addition of ascorbic acid as an extra electron donor extends this pH range, but at pH 6.0 even in the presence of ascorbic acid, transient Δp 's are observed. One way to overcome this problem could be the coreconstitution of the photosynthetic cytochrome *bc*₁ complex with the RC in liposomes. The direct pH-dependent chemical reaction between ubiquinol-0 and cytochrome *c* is then not needed for cyclic electron transport and proton pumping. Initial studies with proteoliposomes containing RCs and photosynthetic cytochrome *bc*₁ complexes indicated that nontransient Δp 's can indeed be generated at lower pHs (W. Crielaard and N. Gabellini, unpublished).

ACKNOWLEDGMENTS

We thank Trees Ubbink-Kok for the isolation of *C. acetobutylicum* membrane vesicles.

This study was supported by the Netherlands Foundation for Chemical Research (SON), which is supported by the Netherlands Organization for the Advancement of Pure Research (NWO).

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