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# Characterization of Protonmotive Force Generation in Liposomes Reconstituted from Phosphatidylethanolamine, Reaction Centers with Light-Harvesting Complexes Isolated from *Rhodopseudomonas palustris*†

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ABSTRACT: Reaction center complexes have been isolated from *Rhodopseudomonas palustris* with either one or both of the light-harvesting complexes attached. Both complexes have been incorporated into liposomes made of phospholipids purified from *Escherichia coli*. Light-driven cycle electron transport could be restored in these proteoliposomes upon the addition of the redox mediators cytochrome *c* and a water-soluble ubiquinone. During cyclic electron transport in this artificial system, protons are extruded electrogenically from the liposomes, and this leads to the generation of a protonmotive force. The optimal conditions for protonmotive force generation were pH 8, a reaction center/lipid ratio of 1.4 nmol/mg, and cytochrome *c* and ubiquinone 0 concentrations of 10 and 400 $\mu$M, respectively. The maximal membrane potential generated under these conditions was −180 mV. From titration studies with a protonophore, it was found that the intrinsic maximal capacity of the reaction centers in protonmotive force generation (the *electro*motive force) equals −210 mV. No evidence was obtained for a contribution of light-harvesting complex II to protonmotive force generation; i.e., these complexes are functionally uncoupled from the reaction centers. Neither was an electrochromic band shift of the carotenoids, present in these complexes, measurable upon illumination. A kinetic model representing the artificial redox chain cytochrome *c*/reaction center/ubiquinone 0 is presented. For this model, data have been used from fast kinetic studies on reaction centers from *Rhodobacter sphaeroides*. The model explains our observation of the discrepancy between the dissociation constant ($K_d$) of reaction centers for cytochrome *c* and the Michaelis constant ($K_M$) for the rate of cytochrome *c* oxidation. The model also explains the light dependency of this $K_M$.

The photosynthetic apparatus of purple non-sulfur bacteria is composed of various pigment/protein complexes. Most of these bacteria contain two light-harvesting complexes [LHI[1] (B870 or B880) and LHII (B800–850)] and the reaction center (RC), which spans the cytoplasmic membrane (Drews, 1985). The light-harvesting pigments absorb light energy, which is subsequently transferred to the reaction center. In the reaction center, the excitation energy induces an oxidation of the bacteriochlorophyll dimer (P870). The liberated electron is transferred very rapidly via a molecule of bacteriopheophytin to the primary and secondary quinones, bound to the reaction center (Parson, 1982). These quinones are subsequently oxidized by an external electron acceptor, which completes the charge-separating photochemical reaction in the reaction center. In vivo, the electrons travel via the $bc_1$ complex to cytochrome $c_2$, which then is used for the re-reduction of P870. The overall result of this cyclic electron flow is the conversion of light energy into an electrochemical proton gradient across the energy-transducing membrane (Dutton et al., 1982). This proton gradient [or protonmotive force ($\Delta p$ or pmf)] can be used to drive a number of energy-requiring processes [for a review, see Hellingwerf and Konings (1985)].

It has been possible to reconstitute isolated reaction centers into liposomes in planar membranes (Schoenfeld et al., 1979; Crofts et al., 1977; Packham et al., 1980; Darszon et al., 1980). Because there is no $bc_1$ complex present in these membranes, it is necessary to supplement the RC liposomes with suitable redox mediators in order to allow light-driven cyclic electron flow (Crofts et al., 1977). Previously (Hellingwerf, 1987a,b), we demonstrated that upon addition of cytochrome *c* and a quinone that is both water and lipid soluble, light-induced unidirectional proton translocation can be observed in a suspension of RC liposomes at relatively alkaline pH values. These liposomes can be useful for several lines of research: (i) coreconstitution or fusion studies in which an *outwardly* directed light-driven proton pump is required; bacteriorhodopsin, which is the only available alternative, usually has a net inwardly directed proton pump and a scrambled orientation [e.g., see Hellingwerf et al. (1978)]; (ii) studies on the effect of a transmembrane electrochemical gradient for protons on the rate of electron transfer of processes in which the reaction center is involved; planar membranes, which have so far been used for these studies, are more difficult to characterize with respect to structure; (iii) experiments in which reactions associated with photosynthetic energy transduction can be studied in vitro, like the electrochromic absorbance changes in the carotenoids upon $\Delta\psi$ generation.

For these studies, it is important to have reaction centers available that (i) can be isolated conveniently, (ii) are free of

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[1] Abbreviations: RC, reaction center; LHI and LHII, light-harvesting complexes I and II, respectively; P870, bacteriochlorophyll dimer; octyl glucoside, *n*-octyl $\beta$-D-glucopyranoside; $UQ_0$, 2,3-dimethoxy-5-methyl-1,4-benzoquinone; $\Delta\psi$, $\Delta$pH, and $\Delta p$ (or pmf), transmembrane gradients of the electrical potential, pH, and electrochemical proton potential, respectively; S-13, 5-chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanilide; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; K-Hepes, potassium *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonate; $TPP^+$, tetraphenylphosphonium ion; SDS–PAA, sodium dodecyl sulfate–polyacrylamide.

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detergent that could impair the energy transduction, and (iii) possess the pigments that show electrochromic absorbance changes. For this reason, we decided to isolate RC complexes from *Rhodopseudomonas palustris*, essentially according to the procedures outlined by Varga and Staehelin (1985). Here we report that these RC's can be reconstituted in liposomes in which they can function as very efficient protonmotive force generators, when supplied with suitable redox mediators. A detailed characterization of this proton pump is presented.

## Experimental Procedures

*Growth of Cells and Isolation of Intracellular Membranes.* *Rhodopseudomonas palustris* strain NCIB 8288 was grown anaerobically under low light intensity (equidistant from two banks of two 60-W tungsten lamps placed 80 cm apart) in a 20-L flask in the medium described by Sistrom (1960), at 30 °C. Exponentially growing cells ($OD_{660}$ approximately 1) were harvested, washed twice in 10 mM Tris-HCl, pH 7.4, and finally resuspended in this buffer. Cells were broken by three successive passages through a French pressure cell (18 000 psi, 4 °C). Debris was removed by centrifugation (30 min, 27000*g*, 4 °C). Intracellular membranes were collected by ultracentrifugation (1 h, 200000*g*, 4 °C) and resuspended in 10 mM Tris-HCl, pH 7.4, at a concentration of 900 μM bacteriochlorophyll (45 mg/mL protein). Membranes were stored in liquid nitrogen until further use.

*Isolation of Reaction Center Complexes.* The RC complexes were isolated according to a modified procedure from Varga and Staehelin (1985). The complexes were extracted from the membranes with 30 mM *n*-octyl β-D-glucopyranoside (octyl glucoside) (RC/LHI/LHII complexes) or a mixture of 15 mM octyl glucoside and 15 mM deoxycholate (RC/LHI complexes) in 10 mM Tris-HCl, pH 7.4, plus 5 mM EDTA for 30 min at 0 °C. The mixture was vortexed every 5 min during solubilization. Nonsolubilized material was removed by centrifugation (2 min in an Eppendorf centrifuge); 0.3 mL of the supernatant was layered on a 9-mL sucrose gradient with 10–40% sucrose (w/v) in 20 mM Tris-HCl, pH 7.4, and either 30 mM octyl glucoside or 15 mM octyl glucoside plus 15 mM deoxycholate, for RC/LHI/LHII or RC/LHI isolation, respectively. Gradients were centrifuged in a Beckman SW 41 Ti rotor (22 h, 35 000 rpm, 6 °C). Pigmented bands were recovered from the gradient, analyzed spectrophotometrically, and stored in liquid nitrogen.

*Incorporation of Reaction Center Complexes into Reconstituted Liposomes.* Acetone-washed *Escherichia coli* phosphatidylethanolamine (40 mg), dispersed in 2 mL of 20 mM K-Hepes, pH 7.0, containing 50 mM KCl and 30 mM octyl glucoside, was sonicated to clarity under a constant stream of nitrogen gas at 0 °C using a probe-type sonicator (MSE Scientific Instruments, West Sussex, U.K.). After the addition of RC complexes (at the desired RC/lipid ratio), the solution was dialyzed at 4 °C for 20 h against a 500-fold volume of 20 mM K-Hepes, pH 7.0, supplemented with 50 mM KCl (three changes). After dialysis, the liposomes were stored in 1-mL aliquots in liquid nitrogen. Before use, the liposomes were thawed slowly at room temperature and sonicated twice for 3 s at 0 °C with the probe-type sonicator, at maximum power output.

*Sucrose Density Gradient Centrifugation of Liposomes.* Liposomes containing RC's at a ratio of 1.4 nmol/mg of lipid (optimal ratio as described under Results) were prepared as described above, except that a trace of octadecyl-rhodamine B chloride was added to the phospholipids (molar ratio 0.1%); 0.3 mL of the preparation was loaded on a 9-mL continuous sucrose gradient from 10% to 50% sucrose (w/v) in 20 mM K-Hepes, pH 7.5, and 50 mM KCl supplemented with 1 mM EDTA. The gradient was centrifuged in a Beckman SW 41 Ti rotor (22 h, 32 000 rpm, 6 °C). After fractionation, the fluorescence (excitation wavelength 560 nm, emission wavelength 590 nm), the absorption at 850 nm, and the refractory index were measured.

*Measurement of* $\Delta\psi$. The $\Delta\psi$ was calculated from the distribution of tetraphenylphosphonium ion ($TPP^+$) across the liposomal membranes. The external $TPP^+$ concentration was measured with a $TPP^+$-sensitive ion-selective electrode (Shinbo et al., 1978; Elferink et al., 1986). A correction for concentration-dependent $TPP^+$ binding to the liposomal membranes was applied according to the model of Lolkema et al. (1982, 1983). The internal volume of reconstituted liposomes were determined by calcein quenching (Oku et al., 1982). Measurements were performed in 20 mM Hepes, 50 mM KCl, and 0.5 mM $MgCl_2$ adjusted to pH 8 or 7 with KOH. The concentration of $TPP^+$ was 2 μM, and except when indicated otherwise, the experiments were carried out in the presence of 20 nM nigericin. The reaction mixture was illuminated from a 150-W projector lamp with fiber optics. The maximal light intensity was 1350 $W/m^2$; when necessary, it was lowered by using neutral density filters. In a typical experiment, 50 μL of a liposome suspension was added to 0.8 mL of buffer. Reduced cytochrome *c* was added from a stock solution of 2 mM 2,3-dimethoxy-5-methyl-1,4-benzoquinone ($UQ_0$) from an ethanolic stock of 100 mM. All experiments were performed in a thermostated vessel (20 °C).

*Measurement of Cytochrome c Oxidation by Reaction Centers.* The oxidation of cytochrome *c* by solubilized RC preparations was monitored in an Aminco DW2a double-beam spectrophotometer equipped with a magnetic stirrer, at 540 minus 550 nm. The cuvette was side-illuminated with red light (wavelength >650 nm). Reaction conditions were the same as described above for the $\Delta\psi$ measurements, except that $TPP^+$ and nigericin were omitted. An extinction coefficient for cytochrome *c* of 19 500 $M^{-1}\cdot cm^{-1}$ was used. The experiments were performed at pH 8.0 and room temperature. Light intensity was varied with neutral density filters, up to a maximum of approximately 40 $W/m^2$. Measurements were taken in quadruplicate, and kinetic parameters were calculated by using nonlinear least squares.

*Analytical Methods.* Protein was determined by the method of Bradford (1976). Bacteriochlorophyll was determined by the method of Clayton (1966). The RC concentration was determined from the absorption difference at 880 nm between reduced (sodium dithionite) and oxidized (potassium ferricyanide) RC's. An extinction coefficient of 113 $mM^{-1}\cdot cm^{-1}$ was used (Clayton, 1966; Feher & Okamura, 1978). Absorption measurements were performed with an Aminco DW2a spectrophotometer (American Instrument Co., Silver Spring, MD). Light intensity was measured with a YSI-Kettering 65 radiometer.

## Results

*Isolation and Optimal Reconstitution of Reaction Centers.* Varga and Staehelin (1985) have described an isolation procedure for RC/LHI and RC/LHI/LHII complexes from *Rhodopseudomonas palustris*. For the solubilization of RC/LHI, they used a combination of octyl glucoside and sodium dodecyl sulfate. As we anticipated that the latter detergent would be very detrimental in subsequent attempts to use these pigment/protein complexes as reconstitutable proton pumps, we replaced it with deoxycholate. When octyl glucoside and deoxycholate are both used at 15 mM, almost all the pigmented complexes are solubilized. Subsequent

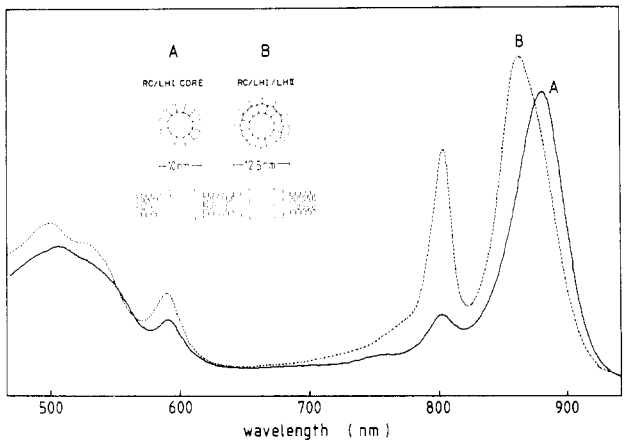


FIGURE 1: Absorption spectra of RC/LHI and RC/LHI/LHII complexes isolated with octyl glucoside/deoxycholate and octyl glucoside, respectively. (A) A sample from the fractions of the sucrose gradient containing RC/LHI complexes was diluted 10-fold in 20 mM Hepes, pH 8, containing 50 mM KCl. The full-scale absorption equals 0.5. The RC concentration in this preparation is 1.2 $\mu$M. (B) The RC/LHI/LHII complexes were diluted 20-fold in the same buffer. The RC concentration in this sample equals 0.35 $\mu$M, and the full scale corresponds to 1 absorption unit. The inset schematically shows the structure of these complexes, as proposed by Varga and Staehelin (1985).

centrifugation on a sucrose gradient reveals a complete separation of RC/LHI and LHII complexes. The two bands are spectroscopically pure (see Figure 1, curve A, for RC/LHI) and are recovered at 1.20 and 1.08 g/cm$^3$, respectively. A minor fraction of the RC/LHI complexes bands at a density of 1.12 g/cm$^3$. The solubilization with only octyl glucoside yielded pure RC/LHI/LHII complexes (Figure 1, curve B). Silver-stained SDS–PAA gels (not shown) revealed patterns similar to those found by Varga and Staehelin (1985) and showed no impurities in the molecular weight range between 15 000 and 100 000.

The isolated RC complexes were reconstituted into liposomes with various lipid mixtures. Initial attempts to reconstitute a functional proton pump [compare Hellingwerf (1987b)] with addition of reduced cytochrome *c* and $UB_0$ made it clear that by far the best results in protonmotive force generation were obtained if phosphatidylethanolamine from *E. coli* was used. The difference was particularly pronounced in comparison with asolectin, isolated from soybean, and phosphatidylcholine from egg yolk, two composite mixtures used frequently in reconstitution studies. For this reason, all subsequent reconstitution experiments were performed with phosphatidylethanolamine from *E. coli*.

Figure 2 shows that initiation of cyclic electron transfer in the reconstituted pump leads to the generation of an electrical potential gradient ($\Delta\psi$) across the liposomal membrane (inside negative) as reflected by the uptake of the lipophilic cation tetraphenylphosphonium. The extent of binding of this lipophilic cation to the reconstituted membranes is relatively small. The dimensionless binding constant [cf. Lolkema et al. (1981)] varied between 5 and 20 for the different batches of liposomes, just as it did in protein-free liposomes, thereby minimizing the corrections that are necessary in the calculation of $\Delta\psi$ [compare de Vrij et al. (1986)]. In the light, the $TPP^+$ accumulation was stable for at least 50 min, under the condition of saturating light intensity (1350 W/m$^2$). The light-dependent $\Delta\psi$ was fully dependent on added cytochrome *c* and $UQ_0$ and sensitive to low concentrations of ionophores like valinomycin. Nigericin dissipates the pH gradient across the liposomal membrane ($\Delta$pH) under the conditions employed, as was confirmed by measurements of pyranine fluorescence (data

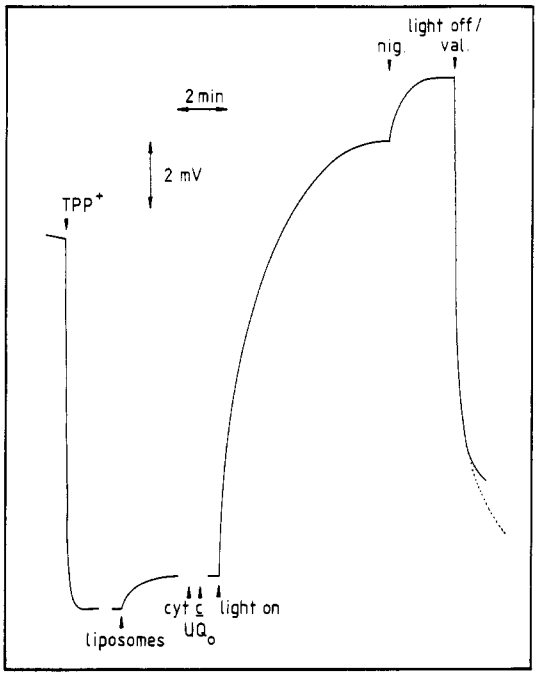


FIGURE 2: Protonmotive force generation in RC liposomes. The calibration shows the response of the $TPP^+$ electrode to a doubling in the concentration of the lipophilic cation (from 1 to 2 $\mu$M); 50 $\mu$L of RC liposomes (with 1.4 nmol of RC/mg of phospholipid) was added as indicated. Cytochrome *c* and $UQ_0$ were added to final concentrations of 10 and 400 $\mu$M, respectively. The suspension was illuminated at the maximal light intensity as indicated. Nigericin and valinomycin were added in final concentrations of 20 and 200 nM, respectively. The dashed line indicates the response after turning off the light.

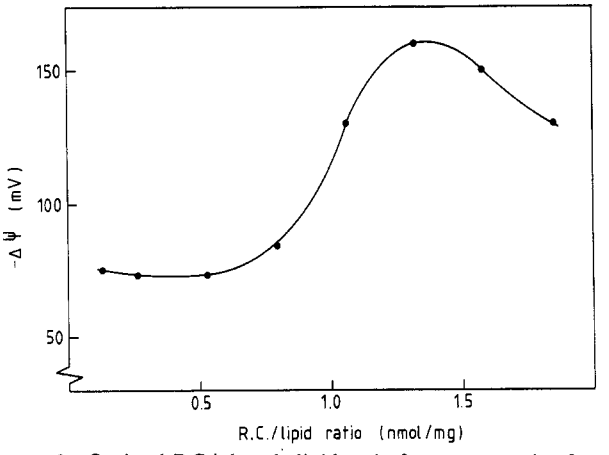


FIGURE 3: Optimal RC/phospholipid ratio for protonmotive force generation. RC liposomes were reconstituted at a fixed lipid concentration and a variable amount of RC's. $\Delta\psi$ was measured at the maximal light intensity as described under Experimental Procedures, at 15 $\mu$M cytochrome *c* and 250 $\mu$M $UQ_0$.

not shown; see below). Concomitantly, nigericin induces a slight increase in $\Delta\psi$, due to the interconversion of the two protonmotive force ($\Delta p$) components. In all subsequent experiments, nigericin is included so that the $\Delta\psi$ values actually equal the total $\Delta p$.

To optimize $\Delta p$ generation by the reconstituted RC proton pump further, the ratio of protein to lipid in the reconstitution was varied. Figure 3 shows that as the relative amount of RC increases the apparent $\Delta p$ generated increases up to a maximum at 1.4 nmol of RC/mg of phospholipid. Assuming an average molecular weight of the lipids of 700, this means a molar ratio of approximately 1000 phospholipids per RC in these liposomes.

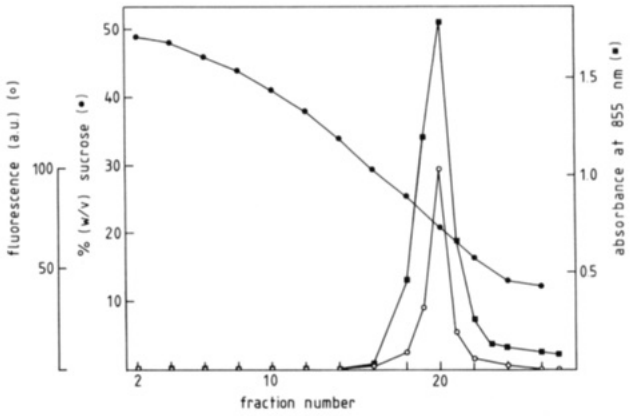


FIGURE 4: Sucrose density gradient centrifugation of RC liposomes. The experimental conditions were as described under Experimental Procedures.

*Characterization of Reconstituted Liposomes.* In order to be able to convert the data on $TPP^+$ uptake in the reconstituted liposomes into values of $\Delta\psi$, it is necessary to have further information about the structure of the liposomes. First, we investigated whether the reconstituted liposomes are homogeneous with respect to the distribution of RC's and lipid over the plane of the membrane. Figure 4 shows the result of equilibrium sucrose density gradient centrifugation of a sample of RC liposomes. The inclusion of a trace amount of fluorescent lipid allowed us to compare the distribution of RC complexes (from the absorbance at 855 nm) over the gradient with the distribution of lipid. The results show that these two components exactly comigrate. Protein-free liposomes would remain at the top of the gradient, whereas solubilized RC's band at 30% sucrose.

A second important structural feature of the reconstituted proteoliposomes is the orientation of the RC's with respect to the plane of the membrane. As RC's are truly intrinsic and transmembrane proteins (Feher & Okamura, 1978), they can be present in either of two orientations: their cytochrome *c* binding site may face the intravesicular or the extravesicular aqueous phase. For convenience, the latter orientation is called the in vivo orientation in this report. The results described in Figure 4 rule out the possibility that a significant part of the RC's in the preparation is present in a third possible configuration, as nonvesicular lipoprotein complexes. The presence of such complexes would lead to an overestimation of the fraction of RC's having the in vivo orientation. The accessibility of the cytochrome *c* binding site of the RC's from the external aqueous phase can be exploited for use in an orientation assay: Flash-induced oxidation of the primary donor of the RC's (P870) will be followed by re-reduction either from cytochrome *c* or, when that electron donor is not available, from the endogenous electron acceptor, the primary or secondary ubiquinone. These two modes of re-reduction display differences in their kinetics (Pachence et al., 1979). A kinetic analysis of the rate of re-reduction of P870, therefore, allows a quantitation of the fraction of the RC's to which reduced cytochrome *c* is available as a reductant. In the proteoliposomes described in this report, this fraction equals the fraction of the RC's with an in vivo orientation. This analysis, as applied to both RC/LHI and RC/LHI/LHII liposomes, leads to the conclusion that in both preparations more than 95% of the RC's have their cytochrome *c* binding site accessible at the external aqueous phase, i.e., have the in vivo orientation. Figure 5 substantiates this conclusion for RC/LHI/LHII liposomes, using kinetic absorbance measurements of P870 oxidation/reduction at 880 nm. Trace A

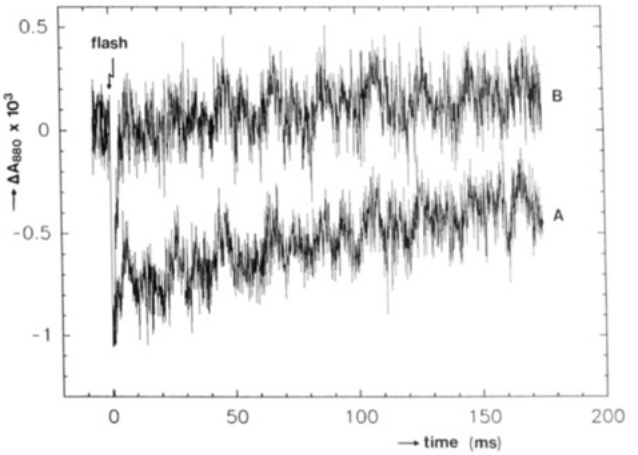


FIGURE 5: Orientation of RC's in RC/LHI/LHII liposomes as determined by kinetic absorbance measurements. RC/LHI/LHII liposomes were prepared as described under Experimental Procedures. Kinetic absorbance measurements were made with a flash kinetic spectrophotometer at 880 nm, as described by Smit et al. (1987). Each flash (from an argon laser) had an intensity of 60 mJ. Routinely, 5–20 transients were averaged. The cuvette had a volume of 250 $\mu$L, and the liposomes were used without dilution. Trace A, 2 $\mu$L of 100 mM $UQ_0$ (50% reduced) was added. Trace B, 6 $\mu$L of 3 mM reduced cytochrome *c* was added. In both traces, 5 transients were averaged.

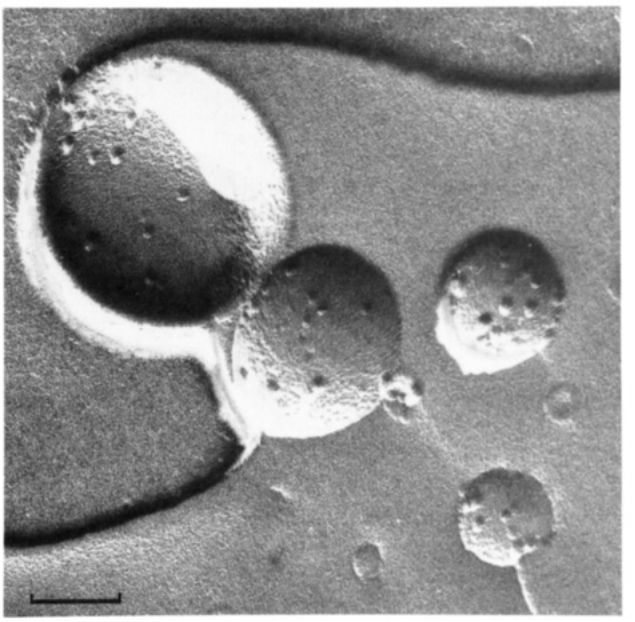

FIGURE 6: Freeze–fracture electron micrograph of reconstituted RC/LHI/LHII liposomes. RC/LHI/LHII liposomes were prepared as described under Experimental Procedures. They were incubated at room temperature in 20 mM Hepes and 50 mM KCl, pH 7, containing, in addition, 10% (w/v) glycerol for 10 min and frozen in freon. For freeze–fracturing, a Balzer's freeze–etch unit was used according to the method described by Moor (1964). The replicas were examined in a Philips EM 300 electron microscope. The bar equals 100 nm.

represents conditions where all the RC's were reduced with $UQ_0H_2$. Under these conditions, upon a flash, P870 is re-reduced from the endogenous quinone electron acceptors of the RC's with a characteristic half-time of about 100 ms. Addition of a high concentration of reduced cytochrome *c* leads to a more rapid re-reduction of nearly all the RC's, with a half-time for reduction of less than 5 ms (trace B). Analogous results were obtained with RC/LHI liposomes.

The reconstituted liposomes were further characterized with respect to structure by freeze–fracture electron microscopy. Figure 6 shows such a result with RC/LHI/LHII liposomes.

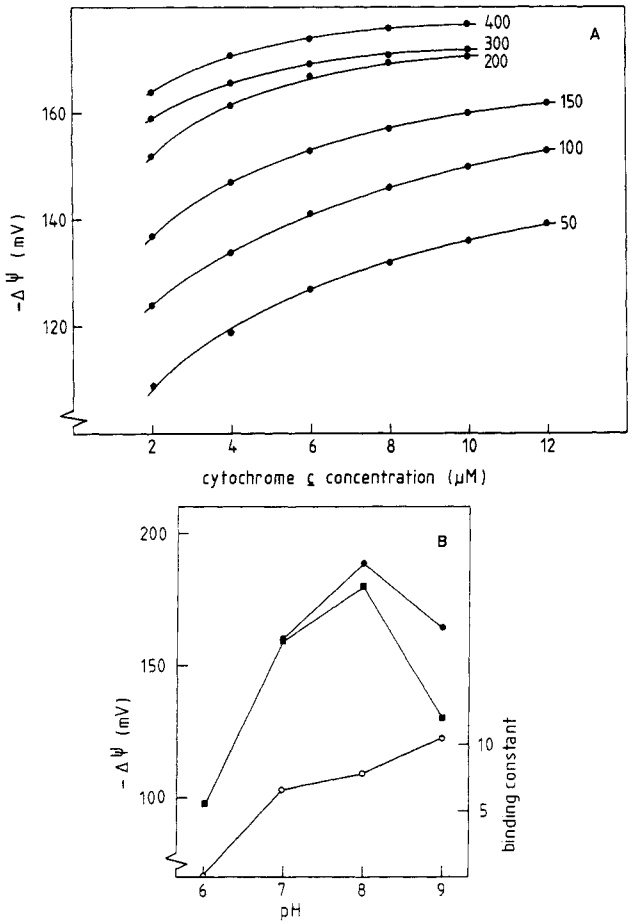


FIGURE 7: Optimization of protonmotive force generation in liposomes reconstituted with RC/LHI/LHII complexes. (A) Optimal concentrations of cytochrome *c* and $UQ_0$; (B) optimal pH. $\Delta\psi$ measurements were performed as described under Experimental Procedures, with liposomes reconstituted at 1.4 nmol of RC/mg of phospholipid. The numbers in panel A indicate the concentration of $UQ_0$ in micromolar. In panel B, standard concentrations of cytochrome *c* and $UQ_0$ were used (see Experimental Procedures). (■) Without nigericin; (●) in the presence of 20 nM nigericin. The open circles represent the dimensionless binding constant for $TPP^+$ (*K*; Lolkema et al., 1982) as determined as a function of the pH.

The particles visible in the fractured liposomes are evenly distributed over the two fracture faces and homogeneously over the liposomes. The size of the liposomes is slightly heterogeneous [compare Hellingwerf (1987a)] and ranges from 50 to 150 nm in diameter. The number of particles in the fracture faces ranges from 0 to 200; their density is in rough agreement with that expected from the lipid to protein ratio during the reconstitution (i.e., 1.4 nmol of RC/mg of lipid). The size of the particles is in agreement with the observations of Varga and Staehelin (1985; i.e., approximately 12 nm). Occasionally, some multilamellar structures were seen among the liposomes.

The internal volume of the reconstituted RC/LHI and RC/LHI/LHII liposomes equals 3 μL/mg of lipid, as determined by calcein quenching (Oku et al., 1982).

*Optimization of the Reconstituted Proton Pump.* As the reconstituted cyclic electron-transfer pathway is dependent on the noncatalyzed reduction of cytochrome *c* by $UQ_0H_2$, it is necessary to optimize the $\Delta p$ generation, with respect to the concentration of these two redox intermediates. Figure 7A shows that at the lowest $UQ_0$ concentrations tested (50 μM), the maximal $\Delta\psi$ generated upon illumination depends hyperbolically on the concentration of cytochrome *c*, such that saturation is only obtained at relatively high cytochrome *c* concentrations. As the $UQ_0$ concentration increases, saturation in cytochrome *c* concentration is observed at lower concentrations such that optimal conditions are obtained when 400 μM $UQ_0$ in combination with 10 μM cytochrome *c* is used. Under these conditions (which are routinely used in subsequent experiments), the reconstituted proton pump shows maximum pmf generation at pH 8 (Figure 7B). If we assume that the two $\Delta p$ components fully interconvert upon addition of nigericin, the ΔpH formed upon illumination is only significant at a pH above 8. This is consistent with the pyranine fluorescence data (see above). Unfortunately, the p*K* of pyranine (7.2; Clement & Gould, 1981) prohibits the experimental demonstration of this ΔpH at very alkaline pH.

The maximal $\Delta p$ generated by a proton pump in a liposome depends on the pumping rate of the proton pump, as well as on the characteristics of passive diffusion of ions through the membrane. Furthermore, it is expected that both pumping rate and passive proton diffusion depend on $\Delta\psi$. The measured maximal $\Delta\psi$ can therefore not be a rigorous measure for the pump's intrinsic capacity to pump protons. An approach to this problem using nonequilibrium thermodynamics has been presented by Westerhoff et al. (1979, 1981). In their description of bacteriorhodopsin, an excellent fit to the experimental results was obtained when it was assumed that (i) both the rate of pumping and the rate of proton (ion) leakage [either through membrane or through protonophore (ionophore)] are linear with their thermodynamic driving force (i.e., for passive proton fluxes the $\Delta p$) and (ii) each photon provides the pump with a fixed amount of free energy which is dictated by the energy levels of the ground state and first excited singlet state of the pump or by other molecular characteristics. Excess energy due to a nonoptimal wavelength of the photon is immediately dissipated via intramolecular vibrations. When expressed on a molar basis, this amount of energy equals the *electro*motive force of the pump, which is therefore independent of both wavelength and light intensity. If an opposing $\Delta p$ of exactly the same magnitude as the *electro*motive force would be present, this would stop the pump in further turnover. Lower values of the $\Delta p$ will only slow down the pump. Here, we assume that the same description of a fixed amount of free energy delivered by each photon also fits the proton pump of the reaction centers.

The *electro*motive force can be determined by a titration with light and a protonophore, which allows an extrapolation to the hypothetical situation of infinite resistance of the membrane to passive proton diffusion. In a plot of the inverse of $\Delta\psi$ against protonophore concentration, a straight line is expected for every light intensity. All lines should intersect in one point reflecting the *electro*motive force and endogenous permeability of the membrane for protons. The analysis (Figure 8) shows that indeed, to a good approximation, straight lines are obtained that intersect in one point. From this intersection, we calculate that the intrinsic capacity of the RC's to generate a $\Delta p$ is as high as −210 mV. This value is lowered to the values actually measured (which are as high as −170 to −180 mV) due to the endogenous proton permeability of the reconstituted liposomes. This endogenous proton permeability is equivalent to that induced by a 0.4 nM sample of the protonophore 5-chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanilide (S-13).

Addition of potassium ascorbate improves $\Delta p$ generation at lower (≤7) pH values. Figure 9 shows that without this redox intermediate, the re-reduction of cytochrome *c* is very slow at pH 7, in contrast to the situation at pH 8. This can be concluded from the rate of the re-reduction of cytochrome *c* in the dark in a suspension of RC's (50 nM), $UQ_0$ (100 μM),

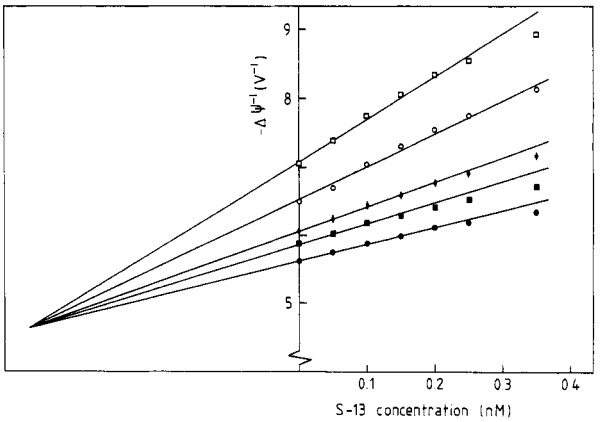


FIGURE 8: *Electro*motive force of RC/LHI/LHII complexes. Measurements were performed under standard conditions as described under Experimental Procedures. The suspension was illuminated with (●) 1350, (■) 700, (♦) 350, (○) 160, and (□) 80 W/m$^2$. The protonophore S-13 was used at the concentrations indicated. For each light intensity, a straight line through the points (except for the point at the highest S-13 concentration) was calculated by linear regression. The coordinates of the point of intersection shown in the figure are −400 pM and 4.76 $V^{-1}$ (=210 mV). This point was calculated as the mean of the intersections of all possible combinations of two lines. The lines in the figure were drawn by eye.

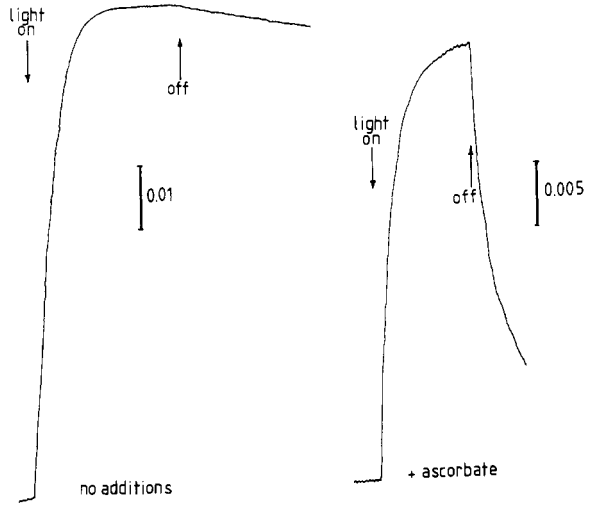


FIGURE 9: Effect of ascorbate on the turnover rate of the proton pump at pH 7.0. Oxidation of cytochrome *c* was measured at 540 minus 550 nm. RC/LHI/LHII complexes were used at an RC concentration of 50 nM, cytochrome *c* concentration of 6 μM, and $UQ_0$ concentration of 100 μM. The standard buffer for Δψ measurements was used at pH 7.0. Ascorbate was added at 0.5 mM. The vertical bars indicate the sensitivity of the absorbance measurements at the wavelength pair indicated above.

and cytochrome *c* (6 μM), after illumination. Therefore, at lower pH, the availability of reduced cytochrome *c* will largely limit the turnover of the light-driven proton pump. The effect of ascorbate, however, is strongly concentration dependent (Figure 10). At low concentrations (up to 0.5 mM), a strong stimulation of the light-dependent Δ*p* is observed, which turns into inhibition upon a further increase in concentration. At pH 8, ascorbate slightly stimulated Δψ formation in the concentration range between 0 and 0.5 mM, with an optimum at about 100 μM. High concentrations of ascorbate caused also at pH 8 a partial Δ*p* dissipation. In the presence of ascorbate at pH 7, the RC's retain the same maximal intrinsic capacity for Δ*p* generation as at pH 8, as was evident from an analysis similar to that shown in Figure 8. The same holds for the intrinsic leakiness of the reconstituted liposomes at pH 7, in the presence of ascorbate. Under these conditions too, the endogenous proton permeability is equivalent to 0.4 nM

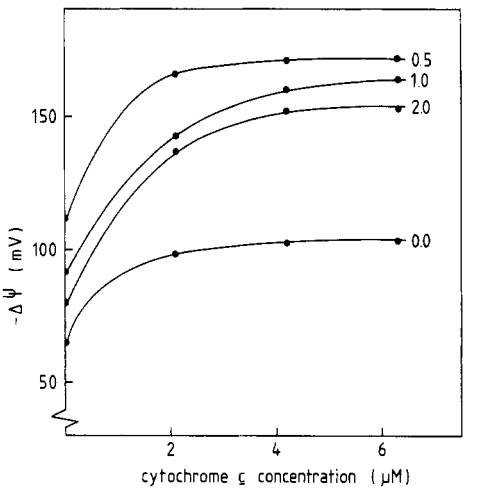


FIGURE 10: Optimization of the reconstituted pump at pH 7.0 with respect to protonmotive force generation in RC/LHI/LHII liposomes. Δψ measurements were performed at pH 7.0 under standard conditions as described under Experimental Procedures. The numbers in the figure indicate the concentration of ascorbate in millimolar.

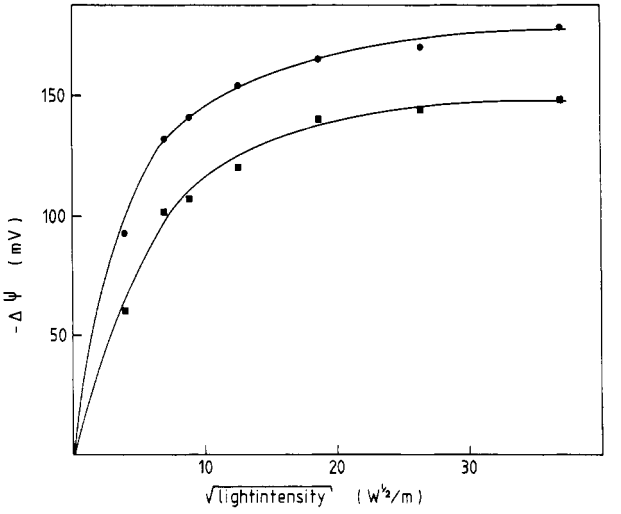


FIGURE 11: Comparison of light saturation of Δψ generation in RC/LHI and RC/LHI/LHII liposomes. The two types of liposomes were reconstituted according to the optimal procedure described under Experimental Procedures, for the RC/LHI/LHII liposomes. This results in a final RC concentration of 0.4 μM. (●) RC/LHI/LHII liposomes; (■) RC/LHI liposomes.

S-13. In the light at pH 7 and with 0.5 mM ascorbate, a steady-state Δψ of around −170 mV can be maintained for at least 20 min in reconstituted RC/LHI liposomes. At pH 6, the turnover of the reconstituted proton pump is severely restricted; nevertheless, Δψ values up to −136 mV were observed in the presence of ascorbate.

*Functional Uncoupling of LHII from RC's.* In intact cells of *Rps. palustris*, light energy, predominantly absorbed by LHII, is channeled to the RC's via LHI. To test whether in isolated form (in RC/LHI/LHII complexes) this functional coupling between RC's and LHII has been retained, Δ*p* generation was assayed as a function of light intensity in two different samples of liposomes. Comparison of Δ*p* generation by reconstituted RC/LHI and RC/LHI/LHII complexes (Figure 11) indicated that this functional coupling was lost, since the light intensity dependence of the generation of Δ*p* in these two preparations is not significantly different. RC/LHI complexes, reconstituted and assayed under the same conditions as optimized for RC/LHI/LHII complexes, generate a slightly lower Δψ. However, the light intensities required for half-maximal Δ*p* generation are 19 and 14 W/m$^2$,

Table I: Estimated Rate Constants for Electron Transfer in Reaction Centers from *Rhodobacter sphaeroides* under Standard Conditions (pH 8, Ionic Strength 50 mM)[a]

| reaction | rate constant | reference |
|---|---|---|
| Cyt $c^{2+}$ + $P^+$ → Cyt $c^{3+}$ + P | $4 \times 10^8$ $M^{-1}\cdot s^{-1}$ | Overfield & Wraight (1980) |
| $Q_A^-Q_B \rightarrow Q_AQ_B^-$ | $6.4 \times 10^3$ $s^{-1}$ | Kleinfeld et al. (1984b) |
| $Q_AQ_B^- \rightarrow Q_A^-Q_B$ | $4.8 \times 10^2$ $s^{-1}$ | Kleinfeld et al. (1984b) |
| $Q_A^-Q_B^- \rightarrow Q_AQ_B^{2-}$ | $8 \times 10^2$ $s^{-1}$ | Kleinfeld et al. (1985) |
| $Q_AQ_B^{2-} \rightarrow Q_A^-Q_B^-$ | 32 $s^{-1}$ | Kleinfeld et al. (1985) |
| $UQ_0 + 2H^+ + Q_B^{2-} \rightarrow UQ_0H_2 + Q_B$ | $1 \times 10^7$ $M^{-1}\cdot s^{-1}$ | Kleinfeld et al. (1984a) |

[a] For an explanation of the symbols, see legend to Figure 13.

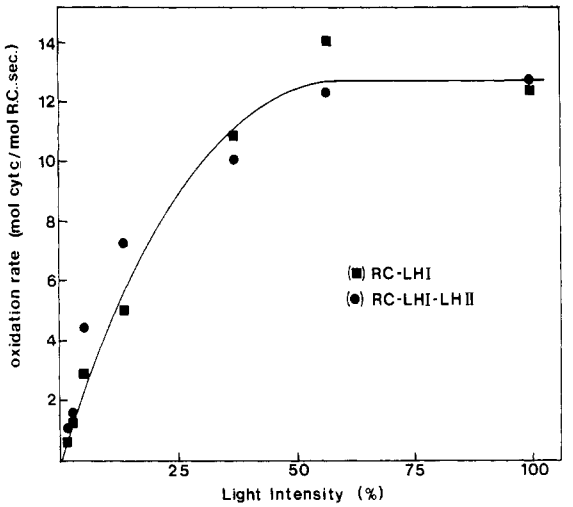


FIGURE 12: Comparison of light saturation of light-dependent cytochrome *c* oxidation by solubilized RC/LHI and RC/LHI/LHII complexes. The RC complexes were diluted 120-fold in standard buffer for absorption measurements (see Experimental Procedures) to 60 nM (for RC/LHI/LHII complexes) and 120 nM (for RC/LHI complexes) RC. Reduced cytochrome *c* and $UQ_0$ were added to final concentrations of 10 and 350 $\mu$M, respectively.

respectively, for RC/LHI and RC/LHI/LHII complexes. The suggestion that LHII is largely functionally uncoupled from the RC's in RC/LHI/LHII complexes is confirmed in a direct assay of the light intensity dependence of the rate of cytochrome *c* oxidation by the RC's (Figure 12). In this assay too, the two complexes do not show a significant difference in their light intensity dependence. Preliminary results of fluorescence emission and excitation spectra confirm the conclusion of a functional uncoupling of LHII from the RC's.

*Kinetics of Cytochrome c Oxidation in the Reconstituted Proton Pump.* The dissociation constant ($K_d$) for binding of cytochrome *c* to RC's of *Rb. sphaeroides*, at an ionic strength equivalent to that of the buffer used for $\Delta\psi$ measurements, equals 5 $\mu$M (Pachence et al., 1983). The Michaelis constant of an enzyme for a substrate is not necessarily equal to the dissociation constant for that substrate. Here, we pose the question how the rate of electron transport catalyzed by the reaction center depends on the concentration of cytochrome *c* and on light intensity. To this purpose, a kinetic model was derived for light-dependent cytochrome *c* oxidation by RC's under the assumption of steady state, using the method of King and Altman (1956). Data for the rates of various electron-transfer reactions were taken from the literature for *Rb. sphaeroides* (see Table I) assuming that RC's from *Rb. sphaeroides* and *Rps. palustris* are very similar. For the measurements and calculations, standard conditions were maintained of 60 nM RC's, 400 $\mu$M $UQ_0$, and pH 8. Cytochrome $c^{2+}$ concentrations were varied from 0.25 to 50 $\mu$M. Since there are large differences in the (pseudo) first-order rate constants for the various electron-transfer reactions within the RC's, a simplified scheme for light-dependent electron

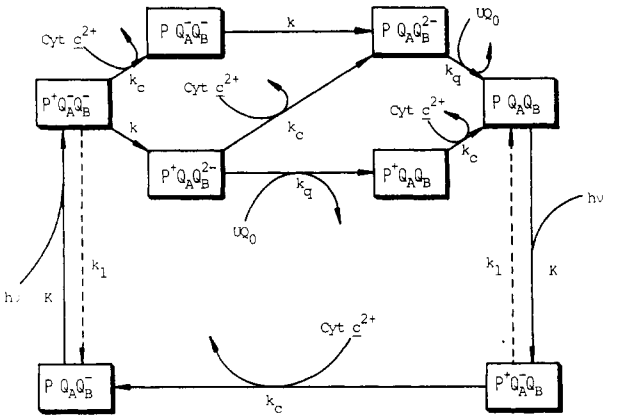


FIGURE 13: Simplified scheme of electron transfer in a suspension of RC's, cytochrome $c^{2+}$, and $UQ_0$. Steps in which cytochrome $c^{2+}$, $UQ_0$, or photons are involved have been modeled as second-order reactions. Symbols for first- or second-order reaction constants are indicated. Their approximate values are the following (from Table I): $k$, 800 $s^{-1}$; $k_c$, $4 \times 10^8$ $M^{-1}\cdot s^{-1}$; $k_q$, $1 \times 10^7$ $M^{-1}\cdot s^{-1}$. The cytochrome $c^{2+}$ concentration was varied from 0.25 to 50 $\mu$M, and the $UQ_0$ concentration was 400 $\mu$M. Under the conditions used, the rate of re-reduction of cytochrome *c* by $UQ_0H_2$ is negligible as could be concluded from absorption measurements after turning off actinic light (an example of this type of measurement is shown in Figure 9). Symbols: P, primary electron donor of the RC's (special pair of bacteriochlorophyll); $Q_A$ and $Q_B$, RC-associated primary and secondary electron-accepting quinone, respectively; $h\nu$, photon.

transfer can be used to describe the reconstituted proton pump (see Figure 13). With these data, the initial equation derived from Figure 13 can be simplified by neglecting small terms:

$$V \approx \frac{V_{max}c}{K_M + c} \tag{1}$$

where

$$V_{max} = KI/2 \tag{2a}$$

and

$$K_M = KI\left(\frac{k_qq + 3k}{2kk_c}\right) + \frac{k_1}{k_c} \tag{2b}$$

$V$ is the turnover rate, $c$ the cytochrome $c^{2+}$ concentration, $q$ the quinone concentration, and $I$ the light intensity. The rate constants in eq 2a and 2b are shown in Figure 13. The approximation (eq 1) was obtained from the initial equation by assuming that (i) the rates $k_1$ and $KI$ are much smaller than $k$, $k_cc$, and $k_qq$, i.e., the "leak process" and photon-induced reduction of $Q_A$ are relatively slow processes, and (ii) the sum of terms containing the two largest exponents in $c$ ($c^3$ and $c^2$) in the initial equation was larger than the sum of the residual terms. It can be calculated that this assumption holds for cytochrome $c^{2+}$ concentrations higher than approximately 1 $\mu$M.

The experimental results show that the initial rate of cytochrome *c* oxidation, upon illumination, obeyed Michaelis–

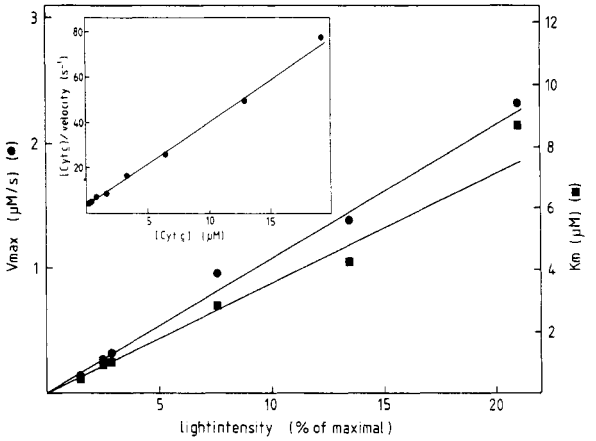


FIGURE 14: Dependence of the $K_M$ and $V_{max}$ for cytochrome *c* oxidation by solubilized RC complexes on the light intensity. The measurements were performed under standard conditions at pH 8.0 and 400 μM $UQ_0$. Inset: An example of a Hanes plot of the rate of cytochrome *c* oxidation by solubilized RC/LHI/LHII complexes. The light intensity was 2.45% of the maximal intensity for absorbance measurements.

Menten kinetics, for both dependence on light intensity and dependence on cytochrome *c* concentration. This allows the definition of a $K_M$ and a $V_{max}$, both for cytochrome *c* and for light intensity. However, the Michaelis–Menten type of kinetics is only predicted (and observed, Figure 14, inset) at not too low cytochrome *c* concentrations and relatively low light intensities. At higher light intensities, the neglected terms containing *KI* in the derivation of eq 1 becomes too large. At light intensities >20% of the maximal intensity, Hanes plots of the rate of cytochrome *c* oxidation became nonlinear due to an increased rate of oxidation at low cytochrome *c* concentrations, as predicted by the model (initial equation). A further prediction was that the $K_M$ and $V_{max}$ for cytochrome *c* oxidation would vary with both $UQ_0$ concentration and light intensity (eq 2a and 2b). Both aspects were experimentally confirmed (for light intensity, see Figure 14). The "offset" for $K_M$ when light intensity approaches zero, predicted in eq 2b, cannot be seen in Figure 14, indicating that $k_1/k_c \ll 1$ μM or that $k_1$ (the first-order rate constant for the "leak" process in Figure 13) is much smaller than 400 $s^{-1}$. This makes the derivation of eq 1 internally consistent.

Using the data on the light intensity dependence of $V_{max}$ and the rate constants of Table I, it is possible to predict the proportionality constant for the light intensity dependence of the $K_M$ for cytochrome *c* oxidation (in relative units of light intensity). This value, $1.8 \times 10^{-8}$ M, is in acceptable agreement with the experimental result, $3.6 \times 10^{-7}$ M. The highest $V_{max}$ value in this experiment (2.3 μM/s) corresponds to 38 electrons/s. Under light saturation, this rate of electron transfer upon illumination increases to 45 electrons/s.

Under the conditions of reconstitution of proton translocation in liposomes, during steady state, cytochrome *c* is largely oxidized. Consequently, the turnover rate of the reconstituted proton pump at pH 8 under light saturation is limited to 7 electrons/s, as was determined by measurements of the rate of cytochrome *c* reduction after switching off the light (analysis similar to that in Figure 9).

## Discussion

We report a modification of the procedure described by Varga and Staehelin (1985) to isolate and reconstitute reaction centers from *Rhodopseudomonas palustris*. Using this method, we obtain a homogeneous liposome population with respect to reaction center distribution; however, the size of the liposomes ranges from 50 to 150 nm in diameter. The particles in the fracture faces of the liposomes are evenly distributed over the two orientations. As the RC's are unidirectionally inserted into the liposomal membrane (cf. Figure 5), we conceive that this means that upon breaking the membrane, the RC has equal probability of ending up in either of the two halves of the membrane. We have shown that it is possible to generate a light-induced protonmotive force (inside negative and alkaline) in these liposomes when they are supplemented with reduced horse heart cytochrome *c* and $UQ_0$. In an earlier report, it was already shown that liposomes containing RC's from *Rhodobacter sphaeroides* extruded protons upon illumination (Hellingwerf, 1984, 1987b). Similar liposomes have been prepared, and shown to be competent in $\Delta p$ generation, with cytochrome *c*/RC/LHI complexes from *Rps. viridis* and with RC/LHI complexes from *Rb. sphaeroides* (data not shown). Qualitative studies on $\Delta p$ generation by reaction center complexes in liposomes have been reported before (Crofts et al., 1977; Darszon et al., 1980). The artificial redox cycle described here has been used previously by Skulachev and collaborators (Drachev et al., 1975; Remmenikov & Samuilov, 1980), for $\Delta p$ generation in chromatophores and intact cells of *Rhodospirillum rubrum*. Preliminary evidence indicates that this artificial cyclic electron-transfer chain (i.e., consisting of a reaction center, cytochrome *c*, and a water-soluble ubiquinone) provides *Rb. capsulatus* mutants, lacking a functional cytochrome $bc_1$ complex, with sufficient energy to restore phototrophic growth (data not shown).

The reconstituted cyclic electron transport can essentially be divided into two reactions. The first reaction, which is driven by light energy absorbed by the RC, is the reduction of ubiquinone by cytochrome *c*. In this reaction, ubiquinone captures two protons from the inside of the liposomes. It then diffuses across the membrane and releases the protons during the energetically favorable reduction of cytochrome *c*. Under the conditions of pH, ionic strength, etc. as described in this report, it was shown (Pachence et al., 1983) that the dissociation constant ($K_d$) of the reaction centers for cytochrome *c* equals 5 μM. For the rates of electron transfer, catalyzed by the RC's in the artificial cyclic electron-transfer system, $K_M$ values for cytochrome *c* are found which are much lower (often below 1 μM, see Figure 7A). The kinetic description of this artificial system for cyclic electron transfer clearly resolves this apparent discrepancy. The $K_M$ for cytochrome *c* is a linear function of the light intensity; i.e., at increasing light intensities, the reaction with cytochrome *c* becomes more rate limiting in the enzymatic reaction cycle. The same holds for the relation between $K_M$ and the $UQ_0$ concentration. In addition, there is a reasonable agreement between the quantitative predictions of the model and experimental results.

Although a small fraction of the RC's may insert into the membrane in an inside-out orientation (with reference to the in vivo situation), the addition, externally, of cytochrome *c* permits only the right side out oriented RC's to take part in cyclic electron transport. This implies that by including cytochrome *c* in the liposomes during preparation one can use the population of inverted RC's for generating an inverted $\Delta\psi$. We found that by using the right side out oriented RC population at pH 7 and pH 8, a maximal $\Delta p$ ($\Delta\psi$) of −170 to −180 mV could be maintained under saturating light intensity. This is exceptionally high compared to other reconstituted pumps [e.g., see Hellingwerf et al. (1985)]. The maximal $\Delta p$ in the light could be maintained for more than 50 min, without any indication of the occurrence of "photo-inhibition" [e.g., see Agel et al. (1987)]. The RC pump therefore promises to be

very useful for studying properties of coreconstituted secondary transport systems. Indeed, in fused membranes of RC liposomes and vesicles from *Streptococcus cremoris*, we were able to measure light-induced amino acid accumulation (Crielaard et al., 1988b).

Titration with light (intensity) and the concentration of a protonophore allow one to extrapolate to a hypothetical situation with an infinitely low proton conductivity of the liposomal membrane (Westerhoff et al., 1979, 1981). Under these conditions, the so-called *electro*motive force of a $\Delta p$-generating pump can be determined (Hellingwerf et al., 1985). For the RC's, a value of −210 mV is obtained, which again is high compared to other pumps, particularly those driven chemically (Driessen et al., 1987). It is difficult to give an exact molecular interpretation of the *electro*motive force of a particular pump. Westerhoff and Dancshazy (1984) suggest that it may be related to the ratio of the rates of a parallel pump and leak reaction. In this respect, it is striking that this ratio of pump and leak rate in the electron-transfer reactions within the RC's is on the order of $10^3$–$10^4$ (Okamura et al., 1982), which would predict an *electro*motive force of −180 to −240 mV (Westerhoff & Dancshazy, 1984).

In preliminary experiments in which we tried to reconstitute RC complexes into asolectin and phosphatidylcholine liposomes, we were not able to generate a high $\Delta\psi$, although the turnover rate of the proton pump was the same as in *E. coli* phospholipid liposomes. Also, an imposed potassium diffusion potential decayed very rapidly in both types of liposomes. This suggests that those liposomes were leaky and that certain lipid-specific interactions with the RC are necessary to form properly sealed liposomes. The endogenous proton permeability of the reconstituted liposomes is very low at pH 7 and 8 (Figure 8). The data of Figure 7B suggest that the ion permeability increases strikingly upon further increase of pH, so that the increased turnover rate of the pump at alkaline pH is not even sufficient to compensate for this increased leakiness. The result is that the maximal $\Delta p$ is reached at submaximal rates of proton pumping [compare Hellingwerf et al. (1987b) and Figure 7B]. The change in proton permeability is accompanied by a change in the ratio of electrogenic permeability over electroneutral proton permeability [cf. the ratio $L_e/L_n$; Westerhoff et al., 1979). The overall result is that a significant $\Delta$pH is only formed at very alkaline pH, at high rates of turnover of the pump. Unfortunately, pyranine is a poor indicator under these circumstances.

Reduced cytochrome *c* had to be added to generate a $\Delta\psi$. No buildup of a $\Delta\psi$ could be observed when the oxidized form of cytochrome *c* was used. This can be explained if one assumes that the electrons from cytochrome *c* make up the main portion of electrons that take part in cyclic electron transport, since ubiquinone is added in the oxidized form, and the amount of electrons coming from the reduced form of the RC's is too small to keep the cycle turning at a sufficiently high rate. It will be interesting to know the results of a redox titration of (i) cytochrome *c* oxidation by the RC's and (ii) light-dependent $\Delta\psi$ generation. Such experiments should give further insight into the factors limiting and regulating the reconstituted proton pump.

At pH 7, the reduction of cytochrome *c* by ubiquinol is most probably the rate-limiting step, since particularly this reaction is known to be pH sensitive and to proceed at decreasing rates with decreasing pH. The standard Gibbs free energy of this reaction decreases from −40 kJ/mol at pH 8 to −29 kJ/mol at pH 7. We measured that in the standard buffer (see Experimental Procedures) the rate of this reaction was 10 times lower at pH 7 and 25 times lower at pH 6, compared to pH 8. In the absence of nigericin, when a pH gradient can develop (Figure 7B), an additional complication must be considered: Ubiquinone, being a weak acid, could accumulate in the alkaline compartment and thereby limit the proton pump also at more alkaline pH. At pH 7, the addition of 0.5 mM ascorbate caused an increase in the maximal $\Delta\psi$. There are two possible explanations for this effect. First, ascorbate might speed up the reaction by increasing the percentage of ubiquinol relatively more than the percentage of reduced cytochrome *c*. A second explanation would be that a linear chain is formed, from ascorbate via cytochrome *c* and the RC to ubiquinone, and that charge separation in the RC plus dissociation and binding of protons alone suffices to cause the increase in $\Delta\psi$. The latter explanation would also imply that, since the quinone pool will become more and more reduced during an experiment, the turnover rate and $\Delta\psi$ will decrease after a few minutes (assuming a turnover rate of 20–40 electrons/s for the RC). This is indeed observed at pH 7. After about 10 min at saturating light intensity, the $\Delta\psi$ slowly declines. Of course, a combination of the two mechanisms cannot be excluded.

In the liposomes prepared from RC/LHI/LHII complexes, the LHII pigments do not significantly transfer excitation energy to the reaction centers. This is in contrast to the LHI pigments which, as indicated by fluorescence emission and excitation spectra (data not shown), we believe do transfer excitation energy to the RC's. In addition, we did not observe a measurable electrochromic carotenoid band shift in these liposomes, in spite of the large potentials. We do not know whether there is a causal relation between these two phenomena. Nevertheless, we think that this type of reconstituted system is well-suited to investigate further the relation between the $\Delta\psi$ measured with the distribution of lipophilic cations and the electrochromic band shifts [compare Crielaard et al. (1988a)].

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