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Functional reconstitution of photosynthetic cyclic electron transfer in liposomes

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The interaction of solubilized reaction centers from the phototrophic bacterium, *Rhodobacter sphaeroides*, and the solubilized ubiquinol–cytochrome *c* oxidoreductase of *Rhodobacter capsulatus* was studied in solution and after coreconstitution in liposomes prepared from *Escherichia coli* phospholipids. Under both conditions, the ubiquinol–cytochrome *c* oxidoreductase increased the light-induced cyclic electron transfer, induced by reaction centers with 2,3-dimethoxy-5-methyl-6-(prenyl)$_2$-1,4-benzoquinone and cytochrome *c* as redox mediators. This effect was more pronounced at acid pH values. The light-induced cyclic electron transfer in these liposomes resulted in the generation of a protonmotive force. Under conditions where the protonmotive force was composed of a membrane potential only, the highest membrane potential (approx. −200 mV) was generated when 2,3-dimethoxy-5-methyl-6-(prenyl)$_{10}$-1,4-benzoquinone was used as redox mediator and when both electron transfer proteins were co-reconstituted in a 2:1 molar ratio. At acid pH non-transitent membrane potentials could be generated only in liposomes containing both reaction centers and the ubiquinol–cytochrome *c* oxidoreductase. These observations show that the pH-dependent direct oxidation of cytochrome *c* by ubiquinol in the liposomes was indeed catalyzed by the ubiquinol–cytochrome *c* oxidoreductase and that this oxidoreductase participates in proton pumping. This could also be concluded from the stimulating effect of 2,3-dimethoxy-5-methyl-6-(prenyl)$_{10}$-1,4-benzoquinone on the membrane-potential-generating capacities in liposomes containing both electron transfer complexes. Such a stimulation was not observed in liposomes containing only reaction centers. The presence of cytochrome *c* in the co-reconstituted system was found to be essential for proton pumping.

Introduction

The photosynthetic apparatus of purple non-sulfur bacteria is composed of various pigment-protein complexes. Most of these bacteria contain two light-harvesting complexes (B875 and B800/850) and the reaction center (RC), which spans the cytoplasmic membrane [1]. 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 rapidly transferred via a molecule of bacteriopheophytin to the primary and secondary quinones (Q_A and Q_B), bound to the reaction center [2]. 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 quinone pool and the bc_1 complex (ubiquinol–cytochrome *c* oxidoreductase) to cytochrome c_2, which is then 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 [3]. This proton gradient (protonmotive force, Δp) can be used to drive a number of energy-requiring processes (for a review, see Ref. 4).

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Abbreviations: RC-LH I, reaction center light-harvesting complex I pigment protein; RC, reaction center; bc_1, ubiquinol–cytochrome *c* oxidoreductase (EC 1.10.2.2); UQ-0, 2,3-dimethoxy-5-methyl-1,4-benzoquinone; UQ-2, 2,3-dimethoxy-5-methyl-6-(prenyl)$_2$-1,4-benzoquinone; UQ-10, 2,3-dimethoxy-5-methyl-6-(prenyl)$_{10}$-1,4-benzoquinone; $\Delta\psi$, membrane potential; ΔpH, transmembrane proton gradient; Δp, transmembrane electrochemical proton potential; TPP^+, tetraphenylphosphonium ion; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; octyl glucoside, *n*-octyl β-D-glucopyranoside.

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Recently [5–7] we have shown that it is possible to reconstitute isolated reaction centers functionally into liposomes. Since a bc_1 complex is not present in these membranes, the RC liposomes need to be supplemented with cytochrome *c* and a water- and lipid-soluble quinone to achieve light-induced proton translocation [5,6] and light-induced Δp generation [7].

These liposomes have been used for: (i) studies in fused membranes in which an outwardly directed light-driven proton pump is required as a driving force for energy-requiring processes [8] (the only available alternative is bacteriorhodopsin, which usually has a scrambled orientation (see, for example, Ref. 9)); (ii) studies on the effect of a transmembrane electrochemical gradient of protons on the rate of electron transfer in processes in which the reaction center is involved [7] – planar membranes, which have so far been used for these studies, are more difficult to characterize with respect to structure; (iii) studies on reactions associated with photosynthetic energy transduction in vitro, like the electrochromic absorbance changes of the carotenoids upon $\Delta\psi$ generation [10].

During these studies, one of the major disadvantages of the exerimental system appeared to be the pH-dependency of the redox reaction between ubiquinol and cytochrome *c* (see, for example, Ref. 8). One way to overcome this problem could be the co-reconstitution with the second component of the photosynthetic electron transfer chain, the bc_1 complex. It has been possible to construct a hybrid system of RCs from *Rhodobacter sphaeroides* and mitochondrial bc_1 complex, which showed light-induced cyclic electron transfer in solubilized form [11] and proton translocation in reconstituted form [12].

In this study we show that a reconstituted hybrid system of *Rb. sphaeroides* RCs and *Rhodobacter capsulatus* bc_1 complex, with UQ-2 or UQ-10 as redox mediator, is able to generate in liposomes a large light-induced protonmotive force, over a broader pH range than with RCs alone.

Materials and Methods

Isolation of reaction center complexes and bc_1 complexes

Rb. sphaeroides 2.4.1 and a green mutant of *Rb. capsulatus* [13,14] were grown photohetrotrophically as described [15,16]. Chromatophores were prepared from *Rb. sphaeroides* according to Ref. 7 and from *Rb. capsulatus* according to Ref. 16. The cytochrome bc_1 complex was isolated from *Rb. capsulatus* chromatophores according to Ref. 17, with the modification that cholate was omitted. Reaction center-light harvesting complex I pigment proteins (RC-LH I complexes) were isolated from chromatophores of *Rb. sphaeroides* using an octyl glucoside/deoxycholate extraction followed by sucrose density centrifugation, according to Ref. 7. Both enzymes were stored in small aliquots in liquid nitrogen.

Reconstitution of RC-LH I complexes and bc_1 complexes into liposomes

Acetone- and diethyl ether-washed *E. coli* phospholipids (type IX, mainly L-α-phosphatidylethanolamine; Sigma St. Louis, MO, U.S.A.) supplemented where indicated with UQ-10, were dispersed in 20 mM potassium-Hepes (pH 7.8)/50 mM KCl/30 mM octyl glucoside. The suspension was sonicated to clarity under N_2 gas at 0 °C using a probe-type sonicator (MSE Scientific Instruments, U.K.) at an output of 2 μm. After the addition of RC-LH I complexes (at 1.4 nmol RC/mg lipid, see Ref. 7) and bc_1 complexes (at the desired RC/bc_1 ratio) the solution was dialyzed at 4 °C for 20 h against a 500-fold volume of 20 mM potassium-Hepes (pH 7.8)/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 an output of 2 μm.

Membrane potential measurements

The membrane potential across the liposomal membrane ($\Delta\psi$) was calculated from the distribution of tetraphenylphosphonium ions (TPP^+) across the liposomal membranes with the Nernst equation. The external TPP^+ concentration was measured with a TPP^+-sensitive ion-selective electrode constructed according to Shinbo et al. [18]. A correction for concentration-dependent TPP^+ binding to the liposomal membranes was applied according to the model of Lolkema et al. [19]. Measurements were performed in 20 mM Hepes/50 mM KCl/0.5 mM $MgCl_2$, adjusted to the desired pH with KOH, supplemented with 2 μM TPP^+. The experiments were carried out in the presence of 20 nM nigericin in order to prevent the possible build-up of a transmembrane pH gradient (ΔpH). Routinely, 50–100 μl of liposomes were added to 1–1.5 ml of buffer. The reaction mixture was illuminated with actinic light from a projector lamp (24 V, 150 W) via a light guide. The maximal light intensity was 1350 W/m^2. All experiments were performed in a vessel maintained at 20 °C.

Measurement of light-induced cytochrome c redox changes

The oxidation of cytochrome *c* by solubilized RC-LH I, RC-LH I/bc_1 preparations and liposomes was monitored at 540–550 nm in an Aminco DW2a double-beam spectrophotometer equipped with a magnetic stirrer. 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 (in the case of solubilized preparations) were omitted. The experiments were performed at room temperature. Light intensity in the cuvette was approx. 40 W/m^2.

Analytical methods

The internal volume (4 μl/mg lipid) of reconstituted liposomes was determined from calceine quenching [20]. Protein was determined according to Lowry et al. [21]. SDS-PAGE was performed according to Laemmli [22]. Reaction center concentrations were calculated from the absorption difference at 865 nm between dithionite-reduced and ferricyanide-oxidized reaction centers [23] using an absorption coefficient of 113 $mM^{-1} \cdot cm^{-1}$ [24]. Cytochrome *b* and c_1 concentrations were estimated according to Ref. 17 using an absorption coefficient of 20 $mM^{-1} \cdot cm^{-1}$ at 561 and 533 nm, respectively. Absorption measurements were performed with an Aminco DW2a spectrophotometer (American Instrument Company, Silver Spring, MD, U.S.A.). Light intensity was measured with a YSI-Kettering No 65 radiometer.

Materials

Octyl glucoside and horse-heart cytochrome *c* were obtained from Sigma. All other materials were reagent grade and were obtained from commercial sources.

Results

The isolation and purification of the photosynthetic reaction center using selective solubilization and sucrose gradient centrifugation have been discussed in detail in [7,10,25]. Analysis of the RC-LH I complexes used for reconstitution by SDS-PAGE and spectroscopy revealed that the composition and degree of purity of the RC-LH I complexes were similar to those described in Refs. 7, 10 and 25. No contaminants of the bc_1-complex subunits could be detected by spectroscopy or SDS-PAGE (not shown). Similar analysis showed that the purified bc_1 complex contained the three catalytic subunits as described previously [17] and was free of photosynthetic pigments (not shown).

Cyclic electron transfer via the bc_1 complex

In order to examine whether the redox cycle containing the photosynthetic reaction center and the photosynthetic bc_1 complex could be created with the purified enzymes, light-induced cytochrome *c* redox changes were followed in the presence and absence of bc_1 complex both in solution and in reconstituted liposomes. Fig. 1 shows the changes of the redox state of cytochrome *c* in the different samples upon switching off the light. After a period of illumination, a steady state between oxidation and reduction of cytochrome *c* was established. Measurement of the initial re-reduction of cytochrome *c* upon switching off the light provides information about the rate of cytochrome *c* oxidation and reduction during light-induced (cyclic) electron transfer, since in the steady state the rate of oxidation equals the rate of reduction. It can be seen that the

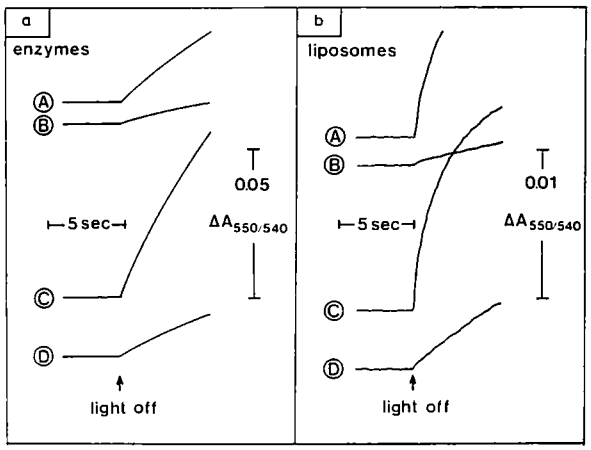

Fig. 1. Light-induced cytochrome *c* redox changes. (Panel a) Solubilized enzymes. Trace A, 780 nM RCs + 390 nM bc_1 complex at pH 7.0; trace B, 780 nM RCs at pH 7.0; trace C, 780 nM RCs + 390 nM bc_1 complex at pH 7.8; trace D, 780 nM RCs at pH 7.8. Experiments were performed in the presence of 400 μM UQ-2 and 20 μM reduced cytochrome *c*. (Panel b) Reconstituted liposomes. Trace A, RC/bc_1 liposomes at pH 6.0; trace B, RC liposomes at pH 6.0; trace C, RC/bc_1 liposomes at pH 8.0; trace D, RC liposomes at pH 8.0. In all cases 50 μl liposomes and 20 μM reduced cytochrome *c* were added to 1 ml buffer. The liposomes contained 40 nmol UQ-10 and (where present) 0.5 nmol bc_1 per nmol RC. Measurements were performed as described in Materials and Methods.

re-reduction of cytochrome *c* was increased both in solution (Fig. 1a) and in liposomes (Fig. 1b) when bc_1 complex was present. The cytochrome *c* reductase activity in solution at high pH was stimulated 4.5-fold by the bc_1 complex (Fig. 1a, traces C vs. D), while after reconstitution (in the presence of UQ-10) the stimulation at high pH was 12.2-fold (Fig. 1b, traces C vs. D). It can also be seen that in all cases the rate of cytochrome *c* reduction was pH-dependent. At low pH in the presence of bc_1 complex the initial rate of cytochrome *c* reduction was decreased both in solution (2.7-fold, Fig. 1a, traces A vs. C) and in liposomes (1.7-fold, Fig. 1b, traces A vs. C). The decrease was, however, more pronounced when only RCs were present, 2.8-fold in solution (Fig. 1a, traces B vs. D) and 2.9-fold in liposomes (Fig. 1b, traces B vs. D). In liposomes (Fig. 1b) the differences in cytochrome *c* rereduction between the redox cycle containing both enzymes and to the cycle containing only the RCs are larger than in solution at both pH values. When both enzymes are present, a considerable rate of cyclic electron transfer is possible at pH 6.0 (Fig. 1b, trace B).

Orientation of both enzymes in liposomes

An important feature of the reconstituted system, with respect to Δp generation, is the orientation of both proteins in the liposomal membrane. Both enzymes can be incorporated in two orientations: with their cytochrome *c* binding site facing the intravesicular or extravesicular aqueous phase (the in vivo orientation).

The accessibility of the cytochrome *c* binding site of the RCs from the external aqueous phase can be exploited for an orientation assay (see Refs. 5–7). Flash-induced oxidation of the primary donor of the RCs (P870) will be followed by re-reduction either from cytochrome *c* or, when this electron donor is not available, from the endogenous electron acceptor, the primary or secondary ubiquinone. These two modes of re-reduction display kinetic differences [26]. A kinetic analysis of the rate of re-reduction of P870, therefore, allows a quantitation of the fraction of the RCs to which reduced cytochrome *c* is available as a reductant. Fig. 2 shows such an analysis for the co-reconstituted liposomes, using kinetic absorbance measurements of P870 oxidation/reduction at 865 nm. Trace A represents conditions where all the RCs were reduced with reduced UQ-0. Under these conditions, upon a flash, P870 was re-reduced from the endogenous quinone electron acceptors of the RCs with a characteristic half-time of about 100 ms (Fig. 2, trace A). Addition of a high concentration of reduced cytochrome *c* led to a more rapid rereduction of nearly all the RCs, with a half-time for reduction of less than 10 ms (Fig. 2, trace B). This leads to the conclusion that more than 90% of the RCs have their cytochrome *c* binding site accessible to the external aqueous phase, i.e., have the in vivo orientation.

A similar analysis can be performed for the orientation of the bc_1 complex. Cytochrome c_1, which in vivo faces the external aqueous phase, can be reduced by ascorbate, which at alkaline pH is fully deprotonated and therefore membrane-impermeable. Insight into the orientation of this enzyme can be obtained by following the oxidation/reduction of cytochrome c_1 by ascorbate in impermeable and permeabilized (Triton X-100-treated) liposomes at 553 nm (α-peak cytochrome c_1). Fig. 3 shows the oxidation/reduction spectra of the co-reconstituted liposomes in the presence and absence of Triton X-100. Trace A shows the difference spectrum between ferricyanide oxidized and ascorbate reduced liposomes. The addition of 1% Triton X-100 to the liposomes leads to a 15% increase in the difference at 553 nm (trace B), indicating that 13% more cytochrome c_1 has become accessible to ascorbate by permeabilization. The controls (traces C, D and E) show that after reduction with a permeable reductant (dithionite, traces C and D) no increase in absorbance at 553 nm can be detected upon solubilization with Triton X-100 (the extra peak at 561 nm results from cytochrome *b* which has also been reduced by dithionite) and that solubilization has no effect on the oxidation by ferricyanide (trace E). Since only 13% of the cytochrome c_1 present in the liposomes is inaccessible to ascorbate in intact liposomes, it is concluded that approx. 87% of the incorporated bc_1 complex is oriented as in vivo.

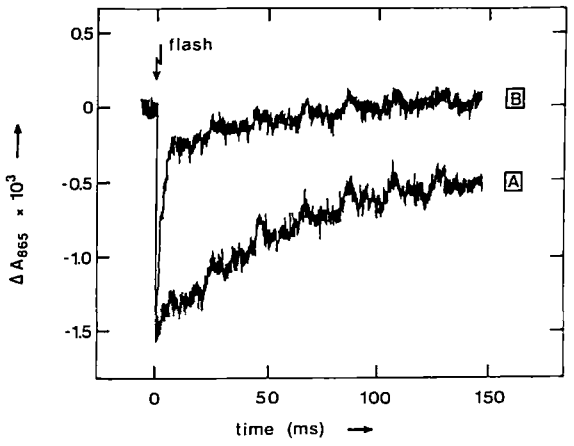

Fig. 2. Orientation of the RCs in RC/bc_1 liposomes (RC/bc_1 molar ratio, 2:1) as determined by kinetic absorbance measurements. Kinetic absorbance measurements were made with a flash-kinetic spectrophotometer at 865 nm, as described by Smit et al. [27]. Each flash (from a laser) had an intensity of 60 mJ. Routinely 5–20 transients were averaged. Cuvette volume was 250 µl and the liposomes were used without dilution. Trace A, in the presence of 800 µM UQ-0 (50% reduced); trace B, in the presence of 70 µM reduced cytochrome *c*. In both traces, ten transients were averaged. Liposomes were prepared as described in Materails and Methods; no UQ-10 was added during reconstitution.

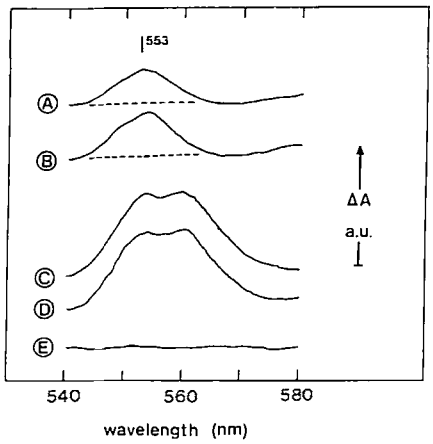

Fig. 3. Orientation of the bc_1 complex in RC/bc_1 liposomes (RC/bc_1 molar ratio, 2:1) as determined by redox difference spectroscopy. Difference spectra: (A) potassium-ferricyanide-oxidized liposomes against ascorbate-reduced liposomes. (B) Potassium-ferricyanide-oxidized solubilized liposomes against ascorbate-reduced solubilized liposomes. (C) Potassium-ferricyanide-oxidized liposomes against dithionite-reduced liposomes. (D) Potassium-ferricyanide-oxidized solubilized liposomes against dithionite-reduced solubilized liposomes. (E) potassium ferricyanide-oxidized liposomes against potassium-ferricyanide-oxidized solubilized liposomes. In all cases 50 µl of liposomes was added to 1 ml 20 mM potassium Hepes (pH 7.8)/50 mM KCl. Solubilization was performed by the addition of 1% Triton X-100. Liposomes were prepared as described in Materials and Methods; no UQ-10 was added during reconstitution (a.u., arbitrary units).

Membrane potential measurements in co-reconstituted liposomes

The generation of a protonmotive force in the reconstituted liposomes was followed in the presence of 20

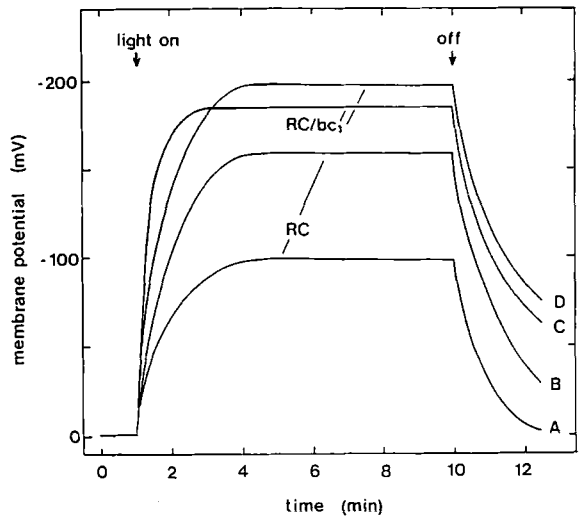

Fig. 4. Light-induced membrane potential generation in reconstituted liposomes. (A) RC liposomes, reconstituted with 40 nmol UQ-10 per nmol RC; (B) RC liposomes, reconstituted without UQ-10, $\Delta\psi$ measurement in the presence of 20 μM UQ-2; (C) RC/bc_1 liposomes (2:1 molar ratio), reconstituted without UQ-10, $\Delta\psi$ measurement in the presence of 20 μM UQ-2; (D) RC/bc_1 liposomes (2:1 molar ratio), reconstituted with 40 nmol UQ-10 per nmol RC. All experiments were performed in the presence of reduced 20 μM cytochrome *c* and 20 nM nigericin. Experiments were performed as described in Materials and Methods.

nM nigericin. Under these conditions only a $\Delta\psi$ could be generated. The ratio of the two enzymes in the membrane for optimum generation of the $\Delta\psi$ was determined. RC/bc_1 ratios were varied between 1:1 and 3:1. The $\Delta\psi$ varied in these liposomes between -160 mV at molar ratios of 1:1 and 3:1, and -200 mV at a molar ratio of 2:1. Since the highest $\Delta\psi$ was observed for a 2:1 RC/bc_1 complex, we used this ratio throughout our study.

The generation of a light-induced $\Delta\psi$ in the (co)-reconstituted liposomes was measured via TPP^+ uptake (Fig. 4). It can be seen that the $\Delta\psi$s generated in the co-reconstituted system were higher than in liposomes containing only RCs (traces C and D vs. A and B), irrespective of the type of ubiquinone used. In liposomes containing only reaction centers, a large $\Delta\psi$ was generated in the light when UQ-2 was used as redox mediator (-168 mV; Fig. 4, trace B). A significantly lower $\Delta\psi$ was generated when UQ-10 was used as redox mediator (-99 mV; Fig. 4, trace A). In contrast, in liposomes containing both enzymes, a higher $\Delta\psi$ was found with UQ-10 than with UQ-2 (-198 vs. -185 mV; Fig. 4, traces D and C).

The effect of UQ-10 on the generation of a $\Delta\psi$ in reconstituted liposomes

UQ-10 is the natural ubiquinone for the bc_1 complex and it is expected that the capacity to generate a high $\Delta\psi$ in the co-reconstituted system in the presence of UQ-10 is due to proton pumping via the bc_1 complex. Proton pumping by RC-UQ-cytochrome *c*, through a chemical ubiquinol–cytochrome *c* reaction (see Refs. 5–7) is expected to be lower with UQ-10 than with UQ-2 due to the lower mobility and water solubility of UQ-10 vs. UQ-2. To prove that the UQ-10 effect was indeed due to proton pumping, the membranes of liposomes containing one or both electron transfer proteins were titrated with UQ-10 and the light-induced generation of a $\Delta\psi$ was followed (Fig. 5). In the absence of UQ-10, no $\Delta\psi$ was generated in either type of liposome. Increasing amounts of UQ-10 resulted, in both types of liposome, in an increase in the $\Delta\psi$. This increase was much larger when both membrane proteins were co-reconstituted (Fig. 5, open symbols vs. closed symbols). Liposomes containing only RCs showed a maximum $\Delta\psi$ generation at approx. 30 nmol UQ-10 per nmol RC (-90 mV) and higher amounts of incorporated UQ-10 led to a slight decrease in the $\Delta\psi$ (Fig. 5, closed symbols). The liposomes containing both RC's and bc_1 complex showed a large increase in $\Delta\psi$ up to -189 mV at 40 nmol UQ-10 per nmol RC (Fig. 5, open symbols). The $\Delta\psi$ decreased when higher amounts of UQ-10 were incorporated; at 160 nmol UQ-10 per nmol RC only a $\Delta\psi$ of -65 mV could be generated. This difference in behaviour with respect to UQ-10 strongly indicated involvement of the bc_1 complex in proton pumping.

Studies with specific inhibitors of the bc_1 complex (antimycin A and myxothiazol) could not provide information about the participation of the bc_1 complex in proton pumping. At a concentration of 1 μM, both inhibitors fully reduced $\Delta\psi$ generation in liposomes containing the complete electron transfer chain, but had also indirect effects on the $\Delta\psi$ generation in liposomes containing only reaction centers.

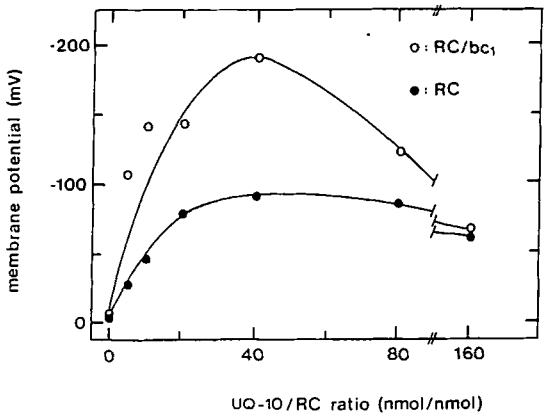

Fig. 5. Effect of increasing amounts of UQ-10 on light-induced membrane potential generation in reconstituted liposomes. (A) RC/bc_1 liposomes (2:1 molar ratio, open circles); (B) RC liposomes (closed circles). All experiments were performed at pH 8.0 in the presence of reduced 20 μM cytochrome *c* and 20 nM nigericin. $\Delta\psi$ measurements were performed as described in Materials and Methods.

The effect of pH on $\Delta\psi$ generation in reconstituted liposomes

$\Delta\psi$ generation in liposomes containing only reaction centers depends heavily on the pH [7]. The electron flow in this system is strictly dependent on the pH-sensitive chemical reaction between cytochrome *c* and ubiquinol. A stable $\Delta\psi$ can be generated only at pH 7 when ascorbate is added as an extra electron donor. At pH 6, even in the presence of ascorbate, only a transient $\Delta\psi$ can be generated [7]. The reaction between ubiquinol and cytochrome *c* catalyzed by the bc_1 complex is also pH-dependent [17], although to a lesser extent (compare Fig. 1). The co-reconstituted system should therefore display different characteristics of $\Delta\psi$ generation at low pH values. The pH profiles of $\Delta\psi$ generation in both types of liposome could thus give more information about the cooperativity of the two membrane complexes in $\Delta\psi$ generation (Fig. 6). At pH 6.0 and pH 7.0 only liposomes containing both enzymes are able to generate a stable $\Delta\psi$ for 10 min (Fig. 6A and B). The $\Delta\psi$ generated under these conditions (−151 mV at pH 6.0 and −174 at pH 7.0) was lower than at pH 8.0 (−195 mV, Fig. 6C). The $\Delta\psi$ in liposomes containing only RCs were transient, with a maximum after 2 min of −40 mV at pH 6.0 (Fig. 6A) and −73 mV at pH 7.0 (Fig. 6B). At pH 8.0, both types of liposome showed a stable $\Delta\psi$ which could be maintained for over 10 min (Fig. 6C). The $\Delta\psi$ generated in the liposomes containing both enzymes was −195 mV, which was again larger than in liposomes containing only reaction centers (−95 mV). Similar pH dependencies in both types of liposome were found when UQ-2 was used as redox mediator (data not shown), but the $\Delta\psi$ generated in liposomes containing only reaction centers were much higher than with UQ-10 (cf. Fig. 4).

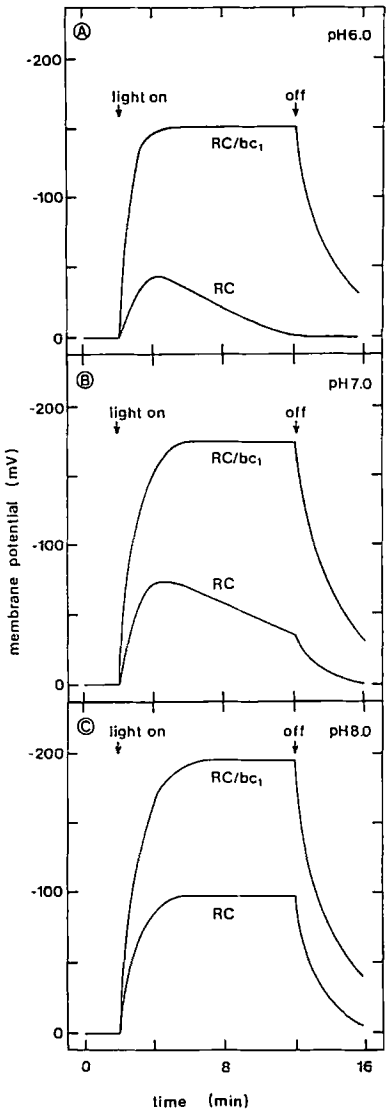

Fig. 6. Effect of pH on light-induced membrane potential generation in reconstituted liposomes. (A) pH 6.0; (B) pH 7.0; (C) pH 8.0. All liposomes were reconstituted in the presence of 40 nmol UQ-10 per nmol RC. RCs and bc_1 complex were reconstituted in a molar ratio of 2:1. $\Delta\psi$ measurements were performed in 20 mM potassium Hepes, containing 50 mM KCl as described in Materials and Methods in the presence of reduced 20 μM cytochrome *c* and 20 nM nigericin.

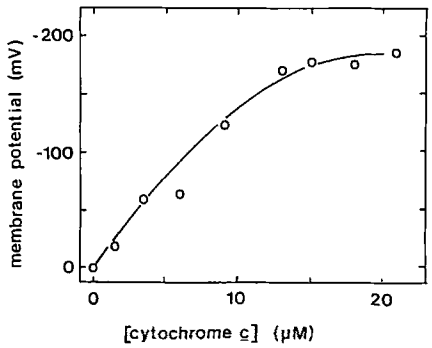

Fig. 7. Cytochrome *c* dependence of the light-induced membrane potential generation in RC/bc_1 liposomes. Liposomes were reconstituted in the presence of 40 nmol UQ-10 per nmol RC. $\Delta\psi$ measurements were performed as described in Materials and Methods at pH 8.0 in the presence of 20 nM nigericin. Cytochrome *c* was added in the reduced form.

Effect of cytochrome c on $\Delta\psi$ generation in RC/bc_1 liposomes

Cytochrome *c* is an obligatory redox intermediate in the redox cycle with RCs and ubiquinones [7]. Prince et al. [28] suggested that RCs and bc_1 complex can perform electron transfer without participation of cytochrome *c*. We investigated the effect of cytochrome *c* on $\Delta\psi$ generation in the co-reconstituted system (Fig. 7). In liposomes containing RCs and bc_1 complexes the $\Delta\psi$ was determined at different concentrations of cytochrome *c*. In the absence of cytochrome *c* no $\Delta\psi$ could be generated in these liposomes. The light-induced $\Delta\psi$ increased with the concentration of cytochrome *c* up to 15 μM.

Discussion

This study shows that it is possible to co-reconstitute functionally in liposomes both integral membrane protein complexes involved in cyclic electron transfer in phototrophic bacteria. It has already been shown that both reaction centers [7] and bc_1 complexes [29] can retain catalytic activity when they are incorporated into liposomes. In this study we have shown that light-driven cyclic electron flow involving both complexes can be reconstructed in detergent solution as well as in liposomes.

As was shown previously [7,8,10], the reconstitution procedure used in this study, using *E. coli* phospholipids and a dialysis buffer containing 50 mM KCl, results in the incorporation of the reaction center complexes in a largely in vivo orientation (cf. Fig. 2). The redox difference studies in Fig. 3 show that the same holds for the bc_1 complex. Addition of cytochrome *c* to the outside of the reconstituted liposomes therefore results in an outward pumping of protons upon illumination (Fig. 4). The $\Delta\psi$ generated through proton pumping (negative inside) is increased by the presence of bc_1 complex in the liposomes, irrespective of the type of redox mediator (UQ-2 or UQ-10) used (Fig. 4), indicating that the proton-pumping capacities of this enzyme are retained after incorporation in liposomes and confirming the predominant in vivo orientation of the enzyme. Proton pumping of the bc_1 complex is optimal when the native quinone UQ-10 is used as redox mediator at a molar ratio of 40 per RC (Fig. 5). Higher amounts of incorporated UQ-10 lead to a decrease in the activity of the enzyme, presumably due to an increase in the redox potential of the UQ pool (cf. Ref. 30), since UQ-10 is added in the oxidized form.

It is shown that cytochrome *c* is an essential component for cyclic electron flow and membrane potential generation in liposomes containing RCs [7] as well as in liposomes containing both RCs and bc_1 complex (Fig. 7). At low concentrations (under 15 μM) cytochrome *c* limits the proton-pumping activity of the hybrid system. No activity can be detected in the absence of cytochrome *c*, indicating that no direct intercomplex electron transfer (cf. Ref. 28) occurs in the liposomes.

The electron transfer via the bc_1 complex is less inhibited at low pH than non-catalyzed electron transfer (Fig. 1), indicating the possibility of using this system for membrane potential generation at low pH. It is not possible to generate high, non-transient membrane potentials in RC liposomes below pH 7.0 (see Ref. 7 and Fig. 6). Co-reconstitution of the bc_1 complex, however, leads to restoration of the membrane-potential-generating capacities at low pH in the liposomes (Fig. 6), presenting direct evidence for proton pumping by this enzyme.

The reduced sensitivity to pH changes of the system under investigation makes it a very attractive proton pump in fused membranes or co-reconstituted systems (cf. Ref. 8). It has the same advantages above the often used redox pumps (see, for example, Ref. 31) as bacteriorhodopsin and the reaction center system used in Ref. 8. The pumping rate (and therefore the generated membrane potential) can be controlled by light intensity. The generated membrane potential is always negative inside, which is not the case in bacteriorhodopsin liposomes (see Ref. 9). The largest advantage compared to the reaction center system described in Refs. 7 and 8 is the fact that now a large membrane potential can be generated over a broad pH range, making the system useful for studies of energy-consuming processes at lower pH values.

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