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Application of thermostable reaction centers from *Chloroflexus aurantiacus* as a protonmotive force generating system

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Reaction centers (RCs) were purified from the thermophilic phototrophic bacterium *Chloroflexus aurantiacus* and reconstituted into liposomes. The dependence of cyclic electron transfer via horse-heart cytochrome *c*, UQ_0 and purified or reconstituted RCs on pH, temperature and ionic strength was investigated. The highest rates of photo-oxidation of cytochrome *c* were achieved at pH 8 or higher, at 55°C and at an ionic strength below $5 \cdot 10^{-4}$. RCs solubilized with octyl β-D-glucoside could be reconstituted by detergent dialysis into liposomes composed of phospholipids from *Escherichia coli* or *Bacillus stearothermophilus*. Upon illumination of RC-containing liposomes in the presence of horse heart cytochrome *c* and UQ_0 a membrane potential of −160 mV was generated. Maximal values of a membrane potential were generated at 1.1 nmol RC/mg phospholipid. RC-containing liposomes were fused with membrane vesicles from *Clostridium fervidus* by a freeze/thaw/sonication method (Driessen et al. (1985) Proc. Natl. Acad. Sci. USA 82, 7555–7559). In these hybrid membranes a protonmotive force of −90 mV could be generated upon illumination. The light-induced protonmotive force could drive uptake of L-serine into the hybrid membranes. Incorporation of this thermostable Δp-generating system into membrane vesicles from bacteria makes it possible to study secondary transport processes under anaerobic conditions.

Introduction

The molecular basis of adaptation to life at high temperatures has been subject of extensive research in the past decade. Several reviews deal with the stability of proteins and other cell components at elevated temperatures [2–4]. Due to subtle differences in hydrogen bonding, disulfide bridges and ionic or hydrophobic interactions proteins of thermophilic micro-organisms are generally more thermostable and thermoactive then those of mesophilic micro-organisms [5–8]. The membranes of thermophilic bacteria are adapted to elevated temperatures by changes in fatty acid and polar headgroup composition of the lipid bilayer [3,9–11]. Thermophilic *Bacillaceae* have been studied most extensively with respect to thermostability of membrane proteins and functional properties of the cytoplasmic membranes (for review see Ref. 12). Hardly anything is known, however, about the functional properties of the cytoplasmic membranes of thermophilic anaerobic bacteria at high temperatures. Studies about the permeability, energy transduction and solute transport in cytoplasmic membranes of thermophilic anaerobes such as *Clostridium fervidus* are hampered by lack of experimental systems suitable for use at high temperatures. Previous studies have demonstrated that functional membrane vesicles can be isolated from *C. fervidus* [13]. Since these membrane vesicles lack a functional Δp-generating system cytochrome *c* oxidase from *Bacillus stearothermophilus* has been incorporated by membrane fusion. In the presence of a suitable electron donor this cytochrome *c* oxidase can act as a Δp generator [13]. A proton-motive force can be obtained for a long period of time and this Δp can drive secondary transport systems up to 50°C. Under these conditions however high evaporation rates and limiting amounts of oxygen restrict transport studies severely (G. Speelmans, unpublished results). The requirement of this model system for oxygen might also

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Abbreviations: $\Delta\psi$, membrane potential; Δp, proton-motive force; $diSC(2)_3$, 3,3′-diethylthiacarbocyanine iodide; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Me_2DodNO, dimethyldodecylamine *N*-oxide; OG, octyl β-D-glucoside; $PhMeSO_2F$, phenylmethansulphonylfluoride; RC, reaction center; SDS-PAA, sodium dodecyl sulphate polyacrylamide; SF-6847, 3,5-di-*tert*-butyl-4-hydroxy-benzelidene malonitrile; TMPD, *N,N.N′,N′*,-tetramethyl-*p*-phenylenediamine; TPP^+, tetraphenylphosphonium ion; UQ_0, 2,3-methoxy-5-methyl-1,4-benzoquinone.

be a disadvantage for studies on transport systems in other strictly anaerobic bacteria. To avoid this problem a Δp-generating system has been developed which can perform under anaerobic conditions. A system based on the reaction centers (RCs) of *Rhodopseudomonas palustris* has been used to study transport in membranes derived from mesophilic anaerobic bacteria [14]. In this communication an analogous system is described with RCs from *Chloroflexus aurantiacus*. The RCs of *C. aurantiacus* have been chosen since the proteins possess a higher thermal stability than RCs of *Rhodobacter sphaeroides* and *R. palustris* [15]. Furthermore, the optimum temperature for proton extrusion in *C. aurantiacus* has been found to be between 67 and 70°C [16]. A method for purification of bold RCs without attached B808–866 light harvesting system has been described [17]. RCs from *C. aurantiacus* have so far not functionally been reconstituted. In this communication we describe the use of the thermostable RCs from *C. aurantiacus* to study transport in fused membranes under anaerobic conditions.

Materials and Methods

Organisms and growth conditions

C. aurantiacus J-10-fl (DSM 635) was grown photoheterotrophically in medium D, containing 2 g/l yeast extract and 1 g/l glycylglycine (pH 8.2–8.4) [18]. Cells were grown at 55°C in completely filled 1 l screw-capped bottles, illuminated with two light bulbs of 15, 75 or 150 watts (low, medium or high light intensity, respectively) which were placed at 15 cm from the bottles. The medium was inoculated with 100 ml of an exponentially growing culture. *Bacillus stearothermophilus* ATCC 7954 was grown at 63°C with vigorous aeration in a medium containing 20 g/l tryptone, 10 g/l yeast extract and 172 mM NaCl (pH 7.0) as described [11]. *C. fervidus* ATCC 43204 was grown anaerobically at 68°C in the TYEG medium as described [13]. Growth rates were determined by measuring the increase in absorbance at 660 nm with a Vitatron 280 colorimeter.

Isolation of cytoplasmic membranes

Cells of *C. aurantiacus* were harvested at the late exponential phase of growth by centrifugation at 18500 $\times g$ for 20 min at 4°C and washed twice with 10 mM Tris/HCl (pH 8.0). The cells were resuspended to a concentration of 0.2 g of cells (wet weight) per ml and disrupted by 2–3 passes through a prechilled French pressure cell at 20000 lb/in^2. Unbroken cells and large debris were removed by centrifugation at 15000 $\times g$ for 20 min at 4°C. The supernatant was again centrifuged at 184000 $\times g$ for 90 min at 4°C. The resulting pellet was washed once with 20 mM Tris/HCl (pH 9.0) and, if not used immediately, stored at −80°C. Membrane vesicles from *C. fervidus* were prepared as described previously [13], rapidly frozen and stored in liquid nitrogen.

Isolation of phospholipids

Phospholipids of *B. stearothermophilus* were isolated using a procedure described by Ames [19]. The obtained crude lipid fraction was acetone/ether-washed according to a procedure of Kagawa and Racker [20]. Crude *E. coli* lipid was also acetone/ether-washed [20].

Isolation of reaction centers

Reaction centers (RCs) of *C. aurantiacus* were isolated by a single DEAE-cellulose chromatography step, using a modification of the procedure described by Shiozawa et al. [17]. The membrane fraction (derived from 50 g wet weight cells) was diluted to a total volume of 0.5 l with 20 mM Tris/HCl (pH 9.0) and solubilized with 1% dimethyldodecylamine *N*-oxide (Me_2DodNO) (w/v) for 1 h at 40°C. Phenylmethansulphonyl fluoride ($PhMeSO_2F$) (1 mM) and sodium ascorbate (1 mM) were added to the membrane suspension. During solubilization, the suspension was stirred slowly (100 rpm) on a magnetic stirrer. After solubilization, the membrane-suspension was cooled and diluted with an equal volume of 1% Me_2DodNO, 20 mM Tris/HCl (pH 9.0). The diluted extract was applied directly to a 3 × 22 cm DEAE-cellulose column (Whatman DE-52), equilibrated with 20 mM Tris/HCl (pH 9.0) and two column volumes of 0.2% Me_2DodNO, 20 mM Tris/HCl (pH 9.0). The column was run at 80–100 ml/h. After the suspension was applied to the column, the material was washed with 2 l of 1% Me_2DodNO, 20 mM Tris/HCl (pH 8.0), followed by 2–4 column volumes of 0.2% Me_2DodNO, 10 mM NaCl, 20 mM Tris/HCl (pH 8.0). Following the washing procedure, the RCs (a blue-grey pigmented band) were eluted from the column with 0.2% Me_2DodNO, 35 mM NaCl, 20 mM Tris/HCl (pH 8.0). The blue-grey pigmented fractions were combined and concentrated by ultrafiltration (Amicon PM10) to a final RC concentration of 30 nmol/ml. This concentrated RC fraction was dialysed overnight against a 500-fold volume of 20 mM Tris/HCl, 0.25 mM sodium ascorbate (pH 8.0) (two changes). RCs were protected from light throughout the isolation procedure. All steps were carried out at 4–6°C. After dialysis the RCs were stored in liquid nitrogen.

Reconstitution of reaction centers

Before reconstitution Me_2DodNO was exchanged for octyl β-D-glucoside. To the RC preparation an equal volume of 2% octyl β-D-glucoside (w/v), 20 mM Tris/HCl (pH 8.0) was added. The suspension was applied to a 3 × 7 cm DEAE-cellulose column (What-

man DE-52), previously equilibrated with 1% octyl β-D-glucoside (OG) (w/v), 20 mM Tris/HCl (pH 8.0). After the RC suspension was applied to the column, the material was washed with 3–4 column volumes of the equilibration buffer (flow rate 40 ml/h). The RCs were eluted with 1% OG (w/v), 250 mM NaCl, 20 mM Tris/HCl (pH 8.0). The combined RC containing fractions were dialysed against a 500-fold volume of 20 mM Hepes/NaOH (pH 8.0). After dialysis, OG was added to a final concentration of 1% (w/v). All steps were carried out at 4–6°C.

E. coli phospholipids (20 mg), dispersed with glass beads in 1 ml of 1% OG (w/v), 20 mM Hepes/NaOH (pH 8.0), were sonicated to clarity with a probe type sonicator (MSE Scientific Instruments, West Sussex, U.K.) at an output of 2 μm. After addition of RCs, up to the desired RC/lipid ratio, the mixture was dialysed against 1000 vols of 20 mM Hepes/NaOH (pH 8.0) at 4°C. After dialysis the liposomes were stored in 1 ml aliquots in liquid nitrogen. The proteoliposomes were thawed slowly at room temperature and the mixture was sonicated twice for 3 s. For reconstitution of *C. aurantiacus* RCs into liposomes prepared from *B. stearothermophilus* phospholipids the same procedure was used except that the dispersed phospholipids as well as the proteoliposomes were sonicated and subsequently dialysed at 40°C.

Fusion of RC-containing liposomes with C. fervidus membrane vesicles

C. fervidus membrane vesicles (1 mg of protein) were mixed with *C. aurantiacus* RC-containing liposomes (containing 10 mg of *E. coli* phospholipid) in a total volume of 1 ml. Fusion was accomplished by the freeze/thaw/sonication method [13]. Fusion efficiency was estimated from the decrease in R_{18} (octadecyl rhodamine β-chloride) fluorescence self-quenching [21]. The membrane preparation obtained after fusion is referred to as hybrid membranes.

Determination of the membrane potential ($\Delta\psi$)

The $\Delta\psi$ was determined from the distribution of the tetraphenylphosphonium ion (TPP^+) across the cytoplasmic membrane, using a TPP^+-sensitive electrode. The $\Delta\psi$ was estimated after correction for concentration-dependent probe binding [22]. The measurements were performed at 30°C in 0.5 mM $MgCl_2$, 2 mM NaCl, 20 mM Hepes/NaOH (pH 8.0), in the presence of 20 nM nigericin, unless stated otherwise. TPP^+ was added to a final concentration of 2 μM. Quinone (UQ_0), horse-heart cytochrome *c* and ascorbic acid were added to final concentrations of 400 μM, 20 μM and 500 μM, respectively. The reaction mixture was illuminated with a projector lamp using fibre optics at a light intensity of 1600 W/m^2. The $\Delta\psi$ was also determined under the same conditions by measuring membrane potential dependent absorbancy changes ($A_{585} - A_{558.6}$) of the probe indicator 3,3′-diethylthiacarbocyanine iodide ($diSC(2)_3$) (8 μM final concentration). The measurements were performed at various temperatures with an Aminco DW2a double beam spectrophotometer with side illumination.

Uptake of amino acid by hybrid membranes

Hybrid membranes containing *C. aurantiacus* RCs (200 μl; 0.20 mg of protein) were added to 0.8 ml of 0.5 mM $MgCl_2$, 2 mM NaCl, 20 mM Hepes/NaOH (pH 7.0). UQ_0, cytochrome *c* and ascorbic acid were added and a protonmotive force (Δp) was generated upon illumination as described above for the membrane potential measurements. All uptakes were performed under anaerobic conditions. Transport (at 35°C) was initiated by adding L-[U-^{14}C]serine (6.4 TBq/mol) to a final concentration of 5.8 μM. At the time intervals indicated samples of 50 μl were removed from the reaction mixture and diluted with 2 ml of ice-cold 0.1 M KCl prior to filtration on cellulose-nitrate filters (0.45 μm pore size). Filters were washed once with 2 ml of 0.1 M KCl. Radioactivity was determined by liquid scintillation spectrometry.

Measurement of cytochrome c oxidation / reduction

The oxidation of horse-heart cytochrome *c* and the reduction by ubiquinol (UQ_0H_2) by solubilized RCs were measured in a 0.5 mM Hepes/NaOH (pH 8.0) and at 40°C, unless indicated otherwise [15]. Light ($\lambda > 650$ nm, 4000 W/m^2) was supplied by a side illumination accessory. A molar extinction coefficient of 19.5 mM^{-1} cm^{-1} for cytochrome *c* was used.

Other methods

Spectra of membrane fractions of *C. aurantiacus* were taken at room temperature using an Aminco DW2a double-beam spectrophotometer. The membrane fractions were diluted with 25% sucrose (w/v), 10 mM Tris/HCl (pH 8.0) and ascorbic acid was added to a final concentration of 0.5 mM. The RC content of the membrane fractions was estimated from the absorbance at 813 nm according to the formula: $0.07 \times A_{860}$ (in membranes) $\times 1.45 = A_{813}$ (RCs in membranes) [23]. The molar extinction coefficients of the RC peaks at 813 and 865 nm are 184 and 135 mM^{-1} cm^{-1}, respectively. Protein concentrations were determined according to Lowry et al. [24], using bovine serum albumin as a standard. SDS polyacrylamide gel electrophoresis was performed as described [25]. Relative amounts of Coomassie blue staining of SDS polyacrylamide gels were estimated by densitometer scanning using a LKB ultroscan XL enhanced laser densitometer. Trapped volume measurements were performed with the fluorophore calcein as described in

[26]. An internal volume of 8 μl/mg protein was determined for the hybrid membranes.

Results

Purification of the reaction centers from C. aurantiacus

The effects of different light intensities on the growth rate and the RC content of the membrane fraction of *C. aurantiacus* were investigated. At low, medium and high light intensities the specific growth rates were 0.018, 0.151 and 0.038 h^{-1}, respectively, whereas the reaction center (RC) contents were 19.2, 6.1 and 12 nmol RC/g cells (wet weight), respectively. Cells grown at low light intensity were used for the isolation of RCs.

A single DEAE-cellulose chromatography step resulted in a 60-fold enrichment of the RCs. The RC fraction obtained contained no other pigments; densitometer scanning of a Coomassie blue stained SDS-PAA gel indicated that the native RC complex accounts for 60–70% whereas the subunits account for 10–15% of the total protein content of the RC fraction (Fig. 1). The A_{280}/A_{813} ratio of different preparations varied between 2.0 and 2.6 which is similar to the purity obtained by Shiozawa et al. [23]. The yield was 35 % and from 50 g cells (wet weight) about 330–365 nmol of RCs were obtained. Further purification steps were not performed, since for reconstitution as a Δp generating system this preparation was sufficiently pure.

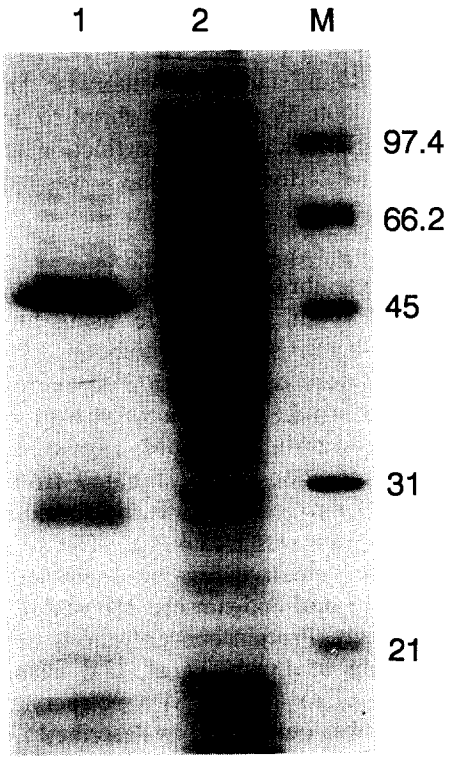

Fig. 1. SDS-PAA gel electrophoresis of partially purified reaction centers of *C. aurantiacus*. Lane 1: the DEAE-cellulose RC-fraction. Lane 2: The membrane fraction. M, molecular mass markers (kilodaltons). The 12.5% PAA gel was stained with Coomassie brilliant blue.

Characterization of the reaction centers

In order to optimize the use of *C. aurantiacus* RCs as a proton-motive force-generating system in membranes, the relevant properties of the protein complex were determined. The pH dependence of cyclic electron transfer by RCs, cytochrome *c* and UQ_0 was determined by following separately the reduction of cytochrome *c* by UQ_0H_2 and the oxidation of cytochrome *c* by RCs (Fig. 2A). The initial rate of cytochrome *c* reduction by UQ_0H_2 increased with increasing pH but remained always lower than the initial rate of cytochrome *c* oxidation by RCs (Fig. 2A). The reduction of cytochrome *c* thus limits the rate of cyclic electron transfer.

The temperature dependency of rates of cytochrome *c* oxidation and reduction are shown in Fig. 2B. From 0 to 50°C cytochrome *c* oxidation and reduction increased. Above 55°C the activity decreased due to thermal inactivation of horse heart cytochrome *c* (data not shown). Reliable rates of oxidation and reduction could therefor not be determined above 55°C. Upon addition of fresh cytochrome *c* to samples heated up to 55°C and then cooled to 45°C full activity of the RCs was found (data not shown).

The dependence of cytochrome *c* oxidation by *C. aurantiacus* RCs on the ionic strength is shown in Fig. 2C. The rate of cytochrome *c* oxidation decreased with increasing ionic strength and this relationship was independent of the salt, $MgCl_2$, KCl or NaCl, used. These results indicate that the ionic strength rather than the interaction with a specific ion caused the reduction of the initial rate of cytochrome *c* oxidation.

Functional reconstitution of the reaction centers

Successful reconstitution of the RCs could only be achieved when octyl β-D-glucoside (OG) was added to the RC-Me_2DodNO mixture before the mixture was applied to the DEAE-cellulose column and when all subsequent elutions were carried out in the presence of OG. When Me_2DodNO was not sufficiently removed it was not possible to generate a membrane potential in the proteoliposomes. After the detergent exchange step the *C. aurantiacus* RCs were functionally reconstituted into liposomes composed of *E. coli* or *B. stearothermophilus* phospholipids by the detergent dialysis method. When *B. stearothermophilus* phospholipids were used the sonication and the dialysis step had to be performed above 40°C to avoid the formation of large aggregates.

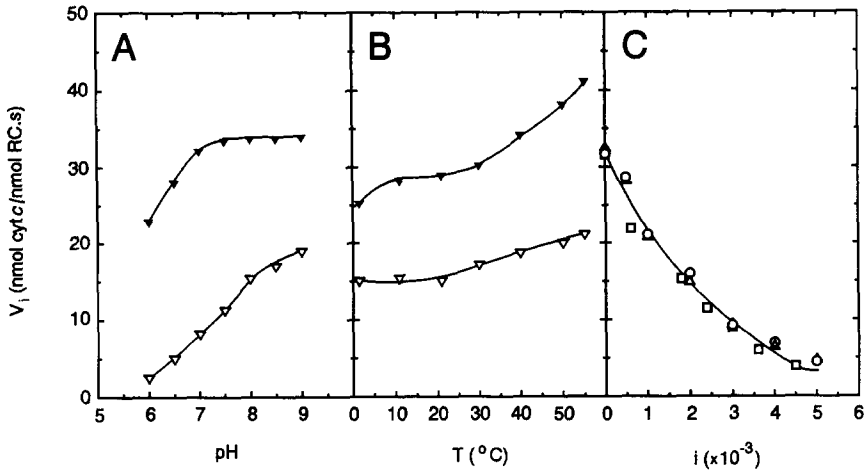

Fig. 2. Effect of pH, temperature and ionic strength on the initial rate of cytochrome *c* oxidation and reduction by solubilized reaction centers. Horse heart cytochrome *c* and UQ_0 were added to final concentrations of 20 μM and 400 μM, respectively. The measurements were performed in 0.5 mM Hepes/NaOH (pH 8.0) at 40°C and at a light intensity of 4000 W/m^2. (A) Effect of pH on (▽) the initial rate of cytochrome *c* reduction and on (▼) the initial rate of cytochrome *c* oxidation. (B) Effect of temperature on (▼) the initial rate of cytochrome *c* oxidation and on (▽) the initial rate of cytochrome *c* reduction. (C) Effect of ionic strength on the initial rate of oxidation of cytochrome *c*. The ionic strength was varied by adding $MgCl_2$ (□), KCl (○) or NaCl (△).

Kinetic parameters of cytochrome c oxidation by the reaction centers

Addition of the redox mediators reduced cytochrome *c* and UQ_0 to soluble RCs under conditions of low ionic strength resulted in a high oxidation rate of cytochrome *c* upon illumination (Table I). At 40°C and pH 8.0, a maximum rate of cytochrome *c* oxidation of approximately 40 electrons per second was reached. This value is comparable to the maximum turnover obtained with RCs of purple bacteria [27]. At 40°C the maximum turnover of the reconstituted RCs was significantly lower than that of soluble RCs (16 electrons/s), while at 25°C the maximum turnover of the reconstituted RCs (13 electrons/s) and the soluble RCs (16 electrons/s) were very similar.

TABLE I

Kinetic parameters of horse heart cytochrome c oxidation by soluble and reconstituted reaction centers from Chloroflexus aurantiacus

The kinetic data were obtained by titration with horse heart cytochrome *c* or UQ_0. The experiments with reconstituted RCs and solubilized RCs were performed at 25°C and 40°C. The K_m values of cytochrome *c* and UQ_0 were determined in the presence of excess UQ_0 and cytochrome *c*.

		K_m (μM)	V_{max} (s^{-1})	
			25°C	40°C
Soluble RC	UQ_0	31.3	n.d. [a]	36.4
	cyt. *c*	1.6	16	42.6
Reconstituted	UQ_0	39.9	13.3	n.d.
	cyt. *c*	7.5	12.7	16

[a] Not determined.

Under the conditions described the RC complex had a high affinity for cytochrome *c*, while the affinity for UQ_0 was one order of magnitude lower. To obtain information about the orientation of the RC in the proteoliposomes, the effect of solubilisation of the RC proteoliposomes with Me_2DodNO on the rate of turnover was studied at 25°C and pH 8.0. No significant effect of Me_2DodNO addition was observed suggesting that most of the RCs were incorporated in the native orientation.

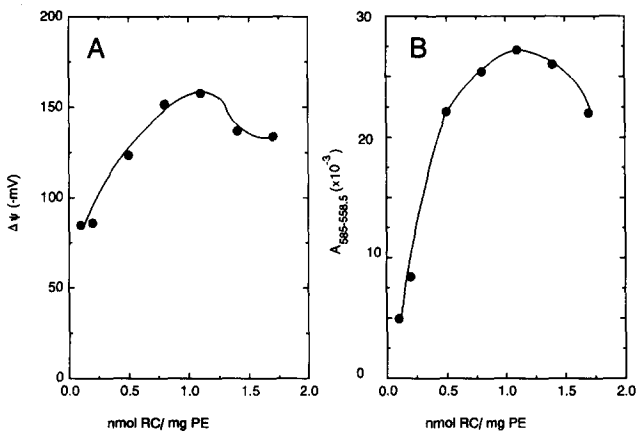

Fig. 3. The effect of the reaction center (RC)/phospholipid ratio on the generation of a membrane potential in RC proteoliposomes. $\Delta\psi$ values were measured in liposomes prepared from *E. coli* phospholipids in which RCs of *C. aurantiacus* were incorporated at different concentrations. Cytochrome *c* (20 μM), UQ_0 (400 μM) and ascorbic acid (500 μM) were added. Measurements were performed in 0.5 mM $MgCl_2$, 2 mM NaCl, 20 mM Hepes/NaOH (pH 8.0) at a light intensity of 1600 W/m^2. (A) TPP^+ electrode measurements performed at 30°C. (B) Absorbance changes of the $diSC(2)_3$ probe performed at 40°C.

Generation of a membrane potential in RC-containing proteoliposomes

To obtain information about the Δp generating capacity of the RC-containing proteoliposomes, the generation of a membrane potential ($\Delta\psi$) by cyclic electron transfer was determined in RC-containing proteoliposomes prepared from *E. coli* or *B. stearothermophilus* phospholipids.

At pH 7.0 or above upon addition of cytochrome *c*, UQ_0, ascorbate and nigericin a $\Delta\psi$ up to -160 mV was generated which was stable for at least 15 min. Below pH 7.0 the $\Delta\psi$ generated was found to be transient. The addition of ascorbic acid was essential for $\Delta\psi$ generation at every pH tested. Up to 45°C the generated $\Delta\psi$ was stable. At temperatures above 45°C the generated $\Delta\psi$ was transient due to leakiness of the membranes. The results were very similar for RC-containing proteoliposomes prepared from *E. coli* and from *B. stearothermophilus* phospholipids.

As expected the generation of $\Delta\psi$ in the RC-containing proteoliposomes was strongly affected by the ionic strength of the reaction mixture. The initial rate of cytochrome *c* oxidation by reconstituted RCs and the generation $\delta\psi$ almost fell to zero upon addition of 6 mM $MgCl_2$ (data not shown).

To optimize $\Delta\psi$ generation in RC proteoliposomes the ratio of RC to lipid was varied. The maximum $\Delta\psi$ value as measured with a TPP^+-electrode at 30°C was generated in proteoliposomes composed of 1.1 nmol RC/mg *E. coli* phospholipid (Fig. 3A). Similar results were obtained at 40°C when the membrane potential indicator probe $diSC(2)_3$ was used to estimate the $\Delta\psi$ in these liposomes (Fig. 3B).

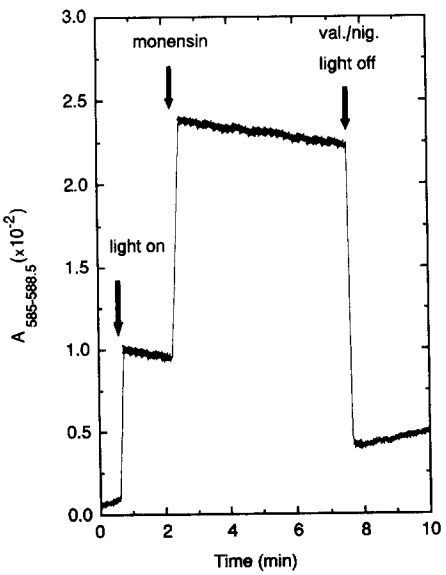

Fig. 4. Generation of a $\Delta\psi$ upon illumination of hybrid membranes obtained by fusing reaction center proteoliposomes with membranes of *C. fervidus*. Experimental conditions were the same as described in the legend to Fig. 3B except that the pH was 7.0 and the temperature 35°C. Valinomycin, nigericin and monensin were added to final concentrations of 2 μM, 2 μM, and 20 nM, respectively.

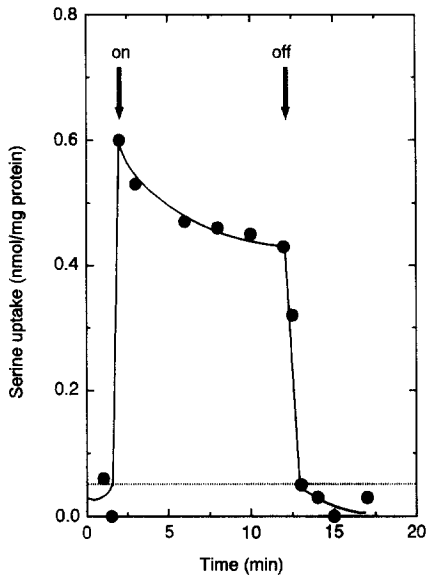

Fig. 5. Light induced uptake of L-serine in hybrid membranes obtained from reaction center proteoliposomes fused with membrane vesicles of *C. fervidus*. The uptake of L-serine was performed under anaerobic conditions in 0.5 mM $MgCl_2$, 2 mM NaCl, 20 mM Hepes/NaOH (pH 7.0) at 35°C. L-[U-^{14}C]-serine was added to a final concentration of 5.8 μM. The dashed line indicates the amount of serine taken up if an equilibration level (1 × accumulation) is reached.

Light-driven amino acid transport in hybrid membranes obtained by fusion of RC-containing liposomes with membrane vesicles of C. fervidus

Fusion of RC-containing liposomes with *C. fervidus* membrane vesicles was accomplished using the freeze/thaw/sonication procedure. The efficiency of the membrane fusion was above 78% as determined by octadecyl rhodamine β-chloride fluorescence quenching assay. Illumination of the hybrid membranes at 30°C and pH 8.0 resulted in a $\Delta\psi$ of approximately -90 mV in the presence of monensin. At pH 7.0 and 35 °C the $\Delta\psi$ was slightly transient and addition of monensin stimulated the $\Delta\psi$ (Fig. 4). At higher temperatures much lower $\Delta\psi$ values were generated. The functional reconstitution of RCs in *C. fervidus* membranes was further demonstrated by the uptake of amino acids by these hybrid membranes upon illumination. The $\Delta\psi$ generated upon illumination by RCs was clearly able to drive the accumulation of L-serine into the hybrid membranes (Fig. 5).

Discussion

The studies presented in this paper demonstrate that purified reaction centers of *C. aurantiacus* can be functionally reconstituted into liposomes. Upon illumination the RCs were able to generate a high proton-

motive force (Δp). After fusion of the RC liposomes with membrane vesicles of *C. fervidus*, light-driven serine uptake could be demonstrated under anaerobic conditions. The method provides us with a tool for studying Δp-dependent processes in isolated membranes that are sensitive to oxygen.

Membrane preparations of *C. aurantiacus* cells grown at low light intensities were used as starting material for the isolation and purification of RCs. In a single DEAE-chromatography step spectroscopically pure RCs with a purity of 80% and with a yield of 35% can be obtained. From 50 g wet weight cells about 330–365 nmol of RCs could be obtained. These results show that these RCs are attractive pumps to be used for generation of a Δp, since they can be obtained by a simple purification procedure in reasonable quantities. Reduced cytochrome *c* is oxidized by soluble RCs at a rather high rate. However, since the purified RCs are devoid of light-harvesting systems (LHs), the maximum turnover is probably limited by the light-trapping capacity of the RCs. The cross-section of a single RC is too small to trap light fast enough to drive the electron transfer at maximum capacity [28]. Therefore it is likely that the rate of cytochrome *c* oxidation can be stimulated by using RCs to which the B808–866 light harvesting system is attached.

Below pH 7 the rate of cytochrome *c* oxidation/reduction by the soluble RCs was severely reduced. This decrease was found to be due to the non-enzymatic reduction of cytochrome *c* by UQ_0H_2 [27]. The limitation set by the pH could possibly be overcome by co-reconstitution of the *C. aurantiacus* RCs with a functional and thermostable bc_1-complex as was shown for the RCs of *Rhodobacter sphaeroides* and the bc_1-complex of *Rhodopseudomonas capsulatus* [29]

For the generation of a $\Delta\psi$ by cyclic electron transfer mediated by RCs ascorbic acid is required. Molenaar et al. [27] made the same observation and discussed the possible interactions between ascorbic acid and the artificial electron transport pathway. Especially at neutral or acidic pH values ascorbate might speed up the reaction by increasing the fraction of reduced UQ_0 relative to the fraction of reduced cytochrome *c*. Another possibility is that a linear chain is formed from ascorbate via cytochrome *c* and the RCs to UQ_0 and that charge separation in the RC plus dissociation and binding of protons alone causes the increase in $\Delta\psi$.

Blankenship et al. [30] showed that both cytochrome *c*-554 and horse-heart cytochrome *c* were slowly photo-oxidized by *C. aurantiacus* RCs. The slow photo-oxidation of horse-heart cytochrome *c* could have been caused by the high ionic strength of the buffers used. The same phenomenon has been encountered in this study. Possibly this problem could be avoided by using native cytochrome *c* as was observed in the cyanobacterium *Anacystis nidulans*. The interaction between horse-heart cytochrome *c* and cytochrome *c* oxidase of this organism was inhibited by increasing ionic strength, while the interaction between the native cytochrome *c*-554 and cytochrome *c* oxidase was enhanced [31]. In buffers with a rather low ionic strength a high $\Delta\psi$ (-160 mV) can be generated upon illumination of RC proteoliposomes. In hybrid membranes of *C. fervidus* membrane vesicles and RC proteoliposomes the $\Delta\psi$ generated upon illumination was significantly increased upon the addition of monensin indicating that a pH gradient is converted into a sodium ion concentration gradient. Both H^+-linked and Na^+-linked transport processes can therefor be studied in hybrid membranes under anaerobic conditions.

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