Application of thermostable reaction centers from Chloroflexus aurantiacus as a protonmotive force generating system
SPEELMANS, G; HILLENGA, D; Poolman, Berend; KONINGS, WN

Published in: Biochimica et biophysica acta
DOI: 10.1016/0005-2728(93)90155-9

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date: 1993

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
Application of thermostable reaction centers from *Chloroflexus aurantiacus* as a protonmotive force generating system

Gea Speelmans, Dirk Hillenga, Bert Poolman and Wil N. Konings

Department of Microbiology, University of Groningen, Haren (Netherlands)

(Received 3 November 1992)

Key words: Reaction center; Reconstitution; Thermophilic; Protonmotive force; (*Chloroflexus aurantiacus*); (*Clostridium fervidus*)

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 $\beta$-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 $\Delta$*p*-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 thermactive than 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 $\Delta$*p*-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 $\Delta$*p* generator [13]. A proton-motive force can be obtained for a long period of time and this $\Delta$*p* 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

Correspondence to: W.N. Konings, Department of Microbiology, University of Groningen, Kerklaan 30, NL-9751 NN Haren, Netherlands.

Abbreviations: $\Delta$*w*, membrane potential; $\Delta$*p*, proton-motive force; diSC$_{2}(3)$, 3,3'-diethyldihexyloxacarbocyanine iodide; Heps, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; Me$_3$DodNO, dimethyldodecylamine N-oxide; OG, octyl $\beta$-glucoside; PhMeSO$_2$F, phenylmethansulphonylfluoride; RC, reaction center; SDS-PAA, sodium dodecyl sulphate polyacrylamide; SF-6847, 3,5-di-tert-butyl-4-hydroxy-benzilidene malonitrile; TMPD, N,N,N',N'-tetramethyl-p-phenylenedia mine; 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 18 500 × 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 20 000 lb/in². Unbroken cells and large debris were removed by centrifugation at 15 000 × g for 20 min at 4°C. The supernatant was again centrifuged at 184 000 × 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% dimethylleucodiamine N-oxide (Me₂DodNO) (w/v) for 1 h at 40°C. Phenylmethylisulphonyl fluoride (PhMeSO₂F) (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% Me₂DodNO, 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% Me₂DodNO, 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% Me₂DodNO, 20 mM Tris/HCl (pH 8.0), followed by 2–4 column volumes of 0.2% Me₂DodNO, 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% Me₂DodNO, 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 Me₂DodNO 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 frac-
tions 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 subse-
quently 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 lipo-
somes (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 R18 (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 (Δψ)
The Δψ was determined from the distribution of the
tetraphenylphosphonium ion (TPP+) across the cyto-
plasmic membrane, using a TPP+-sensitive electrode.
The Δψ was estimated after correction for concentration-
dependent probe binding [22]. The measurements
were performed at 30°C in 0.5 mM MgCl2, 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
(UQ0), horse-heart cytochrome c and ascorbic acid
were added to final concentrations of 5.8 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/m². The Δψ was also
determined under the same conditions by measuring
membrane potential dependent absorbancy changes
(A585 - A558. 6) of the probe indicator 3,3'-diethylthia-
carbocyanine iodide (diSC(2)) (8 μM final concentra-
tion). 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 MgCl2, 2 mM NaCl, 20 mM Hepes/NaOH (pH
7.0). UQ0, cytochrome c and ascorbic acid were added
and a protonmotive force (Δp) was generated upon
illumination as described above for the membrane po-
tential measurements. All uptakes were performed un-
der anaerobic conditions. Transport (at 35°C) was initi-
ated by adding L-[U-14C]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 (UQ0H2) by solubilized RCs
were measured in a 0.5 mM Hepes/NaOH (pH 8.0)
and at 40°C, unless indicated otherwise [15]. Light
(λ > 650 nm, 4000 W/m²) 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 mem-
brane 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 ab-
sorbance at 813 nm according to the formula: 0.07 ×
A860 (in membranes) × 1.45 = A813 (RCs in mem-
branes) [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 deter-
mimed according to Lowry et al. [24], using bovine
serum albumin as a standard. SDS polyacrylamide gel
electrophoresis was performed as described [25]. Rela-
tive amounts of Coomassie blue staining of SDS poly-
acrylamide gels were estimated by densitometer scan-
ing using a LKB ultroscan XL enhanced laser den-
sitometer. Trapped volume measurements were per-
formed with the fluorophore calcein as described in
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 \( \Delta p \) generating system this preparation was sufficiently pure.

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 UQ₉ was determined by following separately the reduction of cytochrome c by UQ₉H₂ and the oxidation of cytochrome c by RCs (Fig. 2A). The initial rate of cytochrome c reduction by UQ₉H₂ 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 therefore 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, MgCl₂, 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-Me₂DodNO 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 Me₂DodNO 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 UQ0 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/m². (A) Effect of pH on (v) 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 (v) 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 MgCl2 (•), KCl (○) or NaCl (△).

Kinetic parameters of cytochrome c oxidation by the reaction centers

Addition of the redox mediators reduced cytochrome c and UQ0 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.

Under the conditions described the RC complex had a high affinity for cytochrome c, while the affinity for UQ0 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 Me2DodNO on the rate of turnover was studied at 25°C and pH 8.0. No significant effect of Me2DodNO addition was observed suggesting that most of the RCs were incorporated in the native orientation.

Table I

<table>
<thead>
<tr>
<th></th>
<th>K_m (μM)</th>
<th>V_max (s^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25°C</td>
<td>40°C</td>
</tr>
<tr>
<td>Soluble RC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UQ0</td>
<td>31.3</td>
<td>n.d. a</td>
</tr>
<tr>
<td>Cyt. c</td>
<td>1.6</td>
<td>16</td>
</tr>
<tr>
<td>Reconstituted</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UQ0</td>
<td>39.9</td>
<td>13.3</td>
</tr>
<tr>
<td>Cyt. c</td>
<td>7.5</td>
<td>12.7</td>
</tr>
</tbody>
</table>

a Not determined.
Generation of a membrane potential in RC-containing proteoliposomes

To obtain information about the $\Delta \psi$ 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, UQ0, 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-containg 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).

**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 B-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 ($\Delta p$). 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 $\Delta p$-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 $\Delta p$, 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$_0$H$_2$ [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.

References