

Ph. D. Thesis

**Effect of nanoscopic environment on
functioning of photosynthetic
reaction center**

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Introduction

Thanks to the structural (crystallography, Nobel prize to Michel, Deisenhofer, Huber) and functional (theory, Nobel prize to Marcus) results together with the works of molecular biology, computer- and electro- techniques, a wealth of information made a relatively clear picture about the kinetics, energetics and stabilization of electron transport within the bacterial photosynthetic reaction center (RC) (see e.g. Sebban *et al.*, 1995; Wraight, 2004). However, several important questions can still be addressed. The physical parameters of the electron transport essentially depend on the type of the RC and environmental factors. From this point of view, the membrane environment is of special interest.

The kinetics and thermodynamics of the electron transfer reactions in RCs are mainly studied in detergents, and the basic processes are already known. However, there are more and more pieces of evidences that these parameters have different features in artificial and/or *in vivo* membranes. The reaction center of *Rhodobacter (Rb.) sphaeroides* consists of three polypeptides, known as L, M and H subunits. The electron transfer after light excitation is initiated by the primary donor bacteriochlorophyll dimer, (P, BChl₂) followed by transient reduction of bacteriochlorophyll monomer (BChl) and bacteriopheophytine (BPheo). Consequently, the electron is trapped by the primary, Q_A, and the secondary quinone, Q_B electron acceptor. Absorbing of one photon in the absence of secondary donor to P⁺ the electron is stabilized in the P⁺Q_AQ_B⁻ state. Depending on the free energy of the Q_B/Q_B⁻ redox couple the charge pair recombines to the ground state PQ_AQ_B with characteristic reaction routes and rates.

If secondary electron donor (e.g. cytochrome *in vivo*, or ferrocene, DAD, TMPD etc. *in vitro*) is present, a second electron can be stabilized on the secondary quinone by accepting two protons in the state of PQ_AQ_BH₂. The doubly reduced and protonated quinone then leaves the RC and is replaced by a fully oxidized quinone, Q, from the membrane pool. This cycle can be repeated until one of the reaction components, the donor or the quinone acceptor, is exhausted. The function of the quinone acceptor complex of the RCs depends both on the redox and binding properties of the quinone molecules (Nagy *et al.*, 2004). If the quinones are bound to the reaction center, their

redox properties are determined by the environment and the chemical identity of the molecule. In this work I offer direct evidence for the role of characteristic native phospholipids - phosphatidylcholine (PC), phosphatidylglycerol (PG), and cardiolipin (CL) - of the membrane in charge stabilization in RC.

The one-dimensional structural and electronic properties of carbon nanotubes have made them suitable candidates for the promotion of heterogeneous electron transfer studies, in which delicate biomolecules “communicate” with the interface of electric circuits. There are convincing pieces of evidence that nanotubes are very efficient components in devices based on biomatter. For example, bioelectrochemical reactions can be driven by attaching small proteins to the surface of carbon nanotubes (Davis *et al.*, 1997; Britto *et al.*, 1996). Well-controlled aligned carbon nanotubes can be applied as immobilization matrices and as mediators for the development of third-generation amperometric biosensor devices (Sotiropoulou and Chaniotakis, 2002). It was found that the protein structure and function were highly influenced by the nanoscale environment. Thirty percent of the activity of the soybean peroxidase and only 1% of the activity of the R-chymotrypsin remained if these proteins were bound to single-walled carbon nanotubes (SWNT) (Karajanagi *et al.*, 2004). The present work is the first to show experiments carried out with photosynthetic RC pigment protein complex, the well-known redox-active enzyme in which light energy initiates a chain of intraprotein electron transport reactions attached to SWNT.

Aims

Determining the role of the lipid bilayer

- 1) Building RCs into liposomes prepared from PC, PG and PC+CL lipids and showing that the liposomes are probably closed bilayer vesicles and the orientation of the RCs are probably random.
- 2) Determining the secondary quinone activity in PC and PG liposomes in terms of the $P^+Q^- \rightarrow PQ$ charge recombination.
- 3) Determining the parameters of components of the $Q_A^-Q_B \rightarrow Q_AQ_B^-$ forward electron transport in PC and PG liposomes.
- 4) Characterizing the binding properties of the anionic lipids (PG and CL) to the RC.

Characterizing the energetics of charge stabilization in lipids

- 5) Determining the free energy difference between the Q_A and Q_B populations in RCs reconstituted in different lipids.
- 6) Characterising the thermodynamic requirements (enthalpy and entropy contribution) of the $Q_A^-Q_B \rightarrow Q_AQ_B^-$ forward electron transport in PC and PG liposomes.

Determining the transmembrane proton gradient

- 7) Incorporating fluorescent dye, pyranine, into the internal compartment of the liposomes and characterising the light induced proton gradient across the lipoprotein bilayer.

Connection RCs to SWNTs

- 11) Preparing SWNT/RC bionanocomposite material and determining the spectral characteristics of this complex.

Materials and methods

Cell cultivation and RC preparations:

Carotenoidless *Rb. sphaeroides* R-26 cells were grown photoheterotrophically under anaerobic conditions in medium supplemented with potassium succinate. Chromatophores and RCs were prepared as described earlier (Tandori *et al.*, 1995). RCs were solubilized by LDAO (*N,N*-dimethyldodecylamine *N*-oxide; Fluka) and purified by ammonium sulfate precipitation followed by DEAE-Sephacel (Sigma) anion-exchange chromatography. The fractions of OD₂₈₀/OD₈₀₃ ratio between 1.27 and 1.50 were collected and used for further experiments.

Proteoliposome preparation:

RC-proteoliposomes were prepared by gel filtration micelle-to-vesicle transition technique. Calculated amount of phospholipid (POPC, Cholesterol, PG, PI) is solved in chloroform and dried on the wall of conical tube under nitrogen stream forming a thin film. The film is dissolved with 0.5 ml of Na-cholate (1.4% solution) or OG (4%) in phosphate buffer (10 μ M KPi, 10 mM KCl, 150 μ M pyranine, pH 7.2). The solution is sonicated in order to form mixed phospholipid/detergent micelles. Small volume of the RC solution is added and is vigorously shaken to allow the phospholipid/protein/detergent micelles formation than loaded on Sephadex G-50 column, previously equilibrated with phosphate buffer (10 μ M KPi, 10 mM KCl, 150 μ M pyranine, pH 7.2). The fraction containing the liposomes was collected and loaded on a second column equilibrated with pyranine free K-phosphate buffer (10 μ M KPi, 10 mM KCl, pH 7.2) in order to remove the external pyranine (Trotta *et al.*, 2002).

Fluorescence measurements:

Pyranine is a water-soluble fluorescent dye which is very sensitive to pH in the interval close to the $pK_a = 7.2$ of the ionization of the hydroxyl group of the molecule in aqueous solution. The ratio between the excitation peaks at 404 and 456 nm can be calibrated and used as an internal pH-indicator regardless the absolute value of the emission peak, allowing to estimate the initial and final pH values in the liposome. The

steady state fluorescence excitation spectra were measured by a Perkin Elmer MPF-44A spectrofluorimeter, which was supplied with a home made sample holder assuring continuous stirring during pH adjustment.

Kinetic absorption spectrophotometry:

Excitation-induced absorption changes were measured routinely by a single-beam kinetic spectrophotometer of local design (Tandori *et al.* 1995; Lakatos *et al.* 2002). The P/P⁺ and Q/Q⁻ redox changes of the primary bacteriochlorophyll dimer and the quinones at the acceptor complex (Nagy *et al.*, 1999; Tandori *et al.*, 1995) and the electrochromic response of the absorption of bacteriopheophytins to the Q_A⁻Q_B and Q_AQ_B⁻ states (Tiede *et al.*, 1996) were detected at 603, 450, and 771 nm, respectively. The apparent one-electron equilibrium constant in the quinone acceptor complex, $K_{AB} = [Q_A Q_B^-] / [Q_A^- Q_B]$, was determined from the rate constants of the fast (k_f) and slow (k_s) components of the P⁺(Q_AQ_B)⁻ → PQ_AQ_B charge recombination in the dark: $K_{AB} = k_f/k_s - 1$. The free energy gap between Q_A⁻Q_B and Q_AQ_B⁻ states is $\Delta G^\circ_{AB} = -k_B T \ln K_{AB}$, where k_B and T are the Boltzmann constant and the absolute temperature, respectively (Wraight and Stein, 1980; Kleinfeld *et al.*, 1985). The quinone binding equilibrium constant of the Q_B site, $K_q = [Q_A Q_B] / [Q_A \dots] = [Q_A^- Q_B] / [Q_A^- \dots]$, was obtained from the model of the oscillation pattern of the semiquinone signal measured at 450 nm upon subsequent flash excitation of the RC (Nagy *et al.*, 1999; Halmschlager *et al.*, 2002). Here [Q_A...] and [Q_A⁻...] denote the concentrations of the RC in oxidized and reduced states of the primary quinone, respectively. These species are able to bind quinone to the temporarily empty Q_B site.

Binding RC to SWNT:

Commercially available SWNTs, produced by a high-pressure CO process (HiPco), have been purchased from Carbon Nanotechnologies Incorporated Company and were purified by wet oxidation technique in the laboratory of László Forró (Institute of Physics of Complex Matter, Ecole Polytechnique Fédérale de Lausanne, Switzerland). In order to bind RCs to SWNTs purified RCs were incubated with the suspension of SWNT and subjected to intensive dialysis against distilled water for three days. The RC concentration was kept as high as possible, routinely 80-100 μM. The mass to mass ratio

was routinely 10 ± 3 mg RC/mg SWNT. After the dialysis the sample was sedimented by ultracentrifuge ($100,000 \times g$, 20 min, SORVALL ULTRA Pro, A-1256 rotor) and the precipitate was suspended in distilled water by sonication for 10-20 seconds. Few drops of sample was dried onto a glass surface under N_2 stream then the optical characteristics were measured.

Data evaluation:

The standard free energy difference of the quinones, ΔG_{AB}^0 was determined from the apparent one-electron equilibrium constant, K_{AB} , in the quinone acceptor complex. The standard enthalpy difference of the quinone states were determined from the slope of the van't Hoff plot of the temperature dependence of the equilibrium constant: $d(\ln K_{AB})/dT = \Delta H^0/RT^2$. The thermodynamic parameters of activation related to the interquinone ET were determined assuming the transition state theory (Eyring's equation):

$$\ln \frac{k}{T} = \ln \frac{\kappa \cdot R}{h} + \frac{\Delta S^\ddagger}{R} - \frac{\Delta H^\ddagger}{R} \cdot \frac{1}{T}$$

where k is the observed rate constant, κ is the transmission coefficient (usually equals to 1, Andreasson *et al.*, 2003), h is the Planck constant, R is the universal gas constant and ΔH^\ddagger and ΔS^\ddagger are the enthalpy and entropy changes of activation, respectively. ΔH^\ddagger is calculated from the slope and ΔS^\ddagger is determined from the intercept of the best-fit of straight lines through the measured data.

New results

The role of the lipid bilayer

- 1) The RCs were built in liposomes prepared from PC, PG and PC+CL lipids, and we have shown that
 - a) the liposomes are probably closed bilayer vesicles;
 - b) the orientation of the RCs are probably random.
- 2) The secondary quinone activity (in terms of the $P^+Q^- \rightarrow PQ$ charge recombination) is larger in PC liposomes (84.4 %) compared to the LDAO detergent (73.9 %), however, this value is only 50 % in PG liposomes. This latter effect can be explained by the accessibility of the Q_B site.
- 3) We determined the components of the $Q_A^- \rightarrow Q_B$ electron transport in PC and PG liposomes.
 - a) There was no difference in the parameters of the fast phases indicating that the mechanism of the intrinsic electron transfer is probably the same in these systems.
 - b) There was large difference in the parameters of the slow components. The rate of the electron transport was facilitated in PC (with positive head group) and reduced in PG (negative head group) compared to the LDAO.
- 4) There was only a limited inhibition of the Q_B site by terbutryn in PG environment.
- 5) The lipids with anionic head groups bound to specific sites of the RC.
 - a) The binding of PG can be well described by Michaelis-Menten kinetics indicating the probably there is only one specific binding site.
 - b) The binding of CL at low concentration does not follow the Michaelis-Menten kinetics.
- 6) The rate of the second electron transfer is also increased both in PC and PG. It is interesting to note that the rate of the first electron transfer is decreased in this latter system.
- 7) The multiple turnover of the RC is determined by the K_{AB} electron equilibrium constant and the K_q quinone equilibrium constant. It seems that K_q is the determining parameter in the membrane environment and it is the smallest in PG.

The energetics of the charge stabilization in lipids

- 8) By measuring the kinetics of the $P^+Q^- \rightarrow PQ$ charge recombination I have determined the free energy difference between the Q_A and Q_B populations. It was -62, -77, -89 mV in LDAO, PC and PG, respectively.
- 9) By using the temperature dependence of K_{AB} I calculated the enthalpy change of the $Q_A^- \rightarrow Q_B$ electron transport and the entropy contribution to this reaction. I have found that
- a) the forward electron transport is driven by the enthalpy change in lipids;
 - b) the enthalpy change is reduced considerably by CL;
 - c) the entropy contribution was small in every lipids compared to the case of the LDAO detergent. It was larger than thermal level ($k^*T=25$ mV/mol) only in LDAO.

Transmembrane proton gradient

- 10) I managed to incorporate fluorescent dye into the internal compartment of the liposomes.
- a) The liposomes prepared this way were stable for long time also at room temperature.
 - b) The proton conductivity remained at the level of about 15-25%, which can be suitable for calculating the different components of the proton motive force after careful calibration.

Connection of RCs to SWNTs

- 11) We prepared SWNT/RC bionanocomposite material and the spectral characteristics of this complex were determined.
- a) The interaction between RCs and SWNTs modifies the electron transport in the RCs specifically resulting the accumulation of positive and negative charges.
 - b) This interaction affects the charge relaxation processes which accompany the electron transport as well.
- c) There is a redox communication between the SWNT and RC after the light excitation, which phenomenon can be a model for several possible practical applications.

References

- Andreasson U, Carlsson T, Andreasson LE (2003) Spectroscopic characterization of a semi-stable, charge-separated state in Cu^{2+} -substituted reaction centers from *Rhodobacter sphaeroides*. *Biochimica et Biophysica Acta-Bioenergetics* **1607**, 45-52.
- Britto PJ, Santhanam KSV, Ajayan PM (1996) Carbon nanotube electrode for oxidation of dopamine. *Bioelectrochemistry and Bioenergetics* **41**, 121-125.
- Davis JJ, Coles RJ, Hill HAO (1997) Protein electrochemistry at carbon nanotube electrodes. *Journal of Electroanalytical Chemistry* **440**, 279-282.
- Halmschlager A, Trotta M, Tandori J, Rinyu L, Pfeiffer I, Nagy L (2002) A mathematical model for quinone-herbicide competition in the reaction centres of *Rhodobacter sphaeroides*. *Functional Plant Biology* **29**, 443-449.
- Kalman L, Gajda T, Sebban P, Maroti P (1997) pH-Metric Study of Reaction Centers from Photosynthetic Bacteria in Micellar Solutions: Protonatable groups equilibrate with the aqueous bulk phase. *Biochemistry* **36**, 4489-4496.
- Karajanagi SS, Kim DY, Kane RS, Dordick JS (2004) Enzyme-nanotube conjugates as functional nanomaterials. *Abstracts of Papers American Chemical Society* **227**, U890.
- Kleinfeld D, Okamura MY, Feher G (1985) Electron transfer in reaction centers of *Rhodospseudomonas sphaeroides*. II. Free energy and kinetic relations between the acceptor states Q_A Qff and $\text{Q}_A\text{Q}_B^{2-}$. *Biochimica et Biophysica Acta* **809**, 291-310.
- Lakatos M, Groma G, Ganea C, Lanyi JK, Varo Gy (2002) Characterization of the azide-dependent bacteriorhodopsin-like photocycle of salinarum halorhodopsin. *Biophysical Journal* **82**, 1687-1695.
- Nagy L, Fodor E, Tandori J, Rinyu L, Farkas T (1999) Lipids affect the charge stabilization in wild-type and mutant reaction centers of *Rhodobacter sphaeroides* R-26. *Australian Journal of Plant Physiology* **26**, 465-473.
- Nagy L, Milano F, Dorogi M, Agostiano A, Laczko G, Szebenyi K, Varo Gy, Trotta M, Maroti P (2004) Protein/lipid interaction in the bacterial photosynthetic reaction center: phosphatidylcholine and phosphatidylglycerol modify the free energy levels of the quinones. *Biochemistry* **43**, 12913-12923.

- Palazzo G, Mallardi A, Giustini M, Berti D, Venturoli G (2000) Cumulant analysis of charge recombination kinetics in bacterial reaction centers reconstituted into lipid vesicles. *Biophysical Journal* **79**, 1171-1179.
- Sebban P, Maroti P, Schiffer M, Hanson DK (1995) Electrostatic dominoes: long distance propagation of mutational effects in photosynthetic reaction centers of *Rhodospirillum rubrum*. *Biochemistry* **34**, 8390-8397.
- Sotiropoulou S, Chaniotakis NA (2002) Carbon nanotube array-based biosensor. *Analytical and Bioanalytical Chemistry* **375**, 103-105.
- Tandori J, Nagy L, Maroti P (1991) Semiquinone oscillation as a probe of quinone herbicide binding in bacterial reaction centers. *Photosynthetica* **25**, 159-166.
- Tandori J, Nagy L, Puskas A, Droppa M, Horvath G, Maroti P (1995) The IleL229 -> Met mutation impairs the quinone binding to the Q_B⁻ pocket in reaction centers of *Rhodospirillum rubrum*. *Photosynthesis Research* **45**, 135-146.
- Tiede DM, Vazquez J, Cordova J, Marone PA (1996) Time-resolved electrochromism associated with the formation of quinone anions in the *Rhodospirillum rubrum* R26 reaction center. *Biochemistry* **35**, 10763-10775.
- Trotta M, Milano F, Nagy L, Agostiano A (2002) Response of membrane protein to the environment: the case of photosynthetic Reaction Centre. *Materials Science & Engineering C-Biometric and Supramolecular Systems* **22**, 263-267.
- Wraight CA (2004) Is the midpoint redox potential of Q_A in bacterial reaction centers pH dependent or independent? *Biophysical Journal* **86**, 12A.
- Wraight CA, Stein RR (1980) Redox equilibrium in the acceptor quinone complex of isolated reaction centers and the mode of action of *O*-phenanthroline. *FEBS Letters* **113**, 73-77.