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pH and Potential Transients of the bc_1 Complex Co-reconstituted in Proteo-Lipobeads with the Reaction Center from *Rb. sphaeroides*

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Abstract

His-tag technology is employed to bind membrane proteins, such as the bc_1 complex and the reaction center (RC) from *Rb. sphaeroides*, to spherical as well as planar surfaces in a strict orientation. Subsequently, both types of surfaces are subjected to in-situ dialysis to form proteo-lipobeads (PLBs) and protein-tethered bilayer membranes (ptBLMs), respectively. PLBs based on Ni-nitrile tri-acetic acid (NTA-) functionalized agarose beads that have a diameter ranging from 50–150 μm are used to assess proton release and membrane potential parameters by confocal laser-scanning microscopy (LSM). The pH and potential transients are thus obtained from bc_1 activated by the RC. To assess the turnover of bc_1 excited by the RC in a similar setting, we used

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ASSOCIATED CONTENT

Supporting Information. Additional results, such as measurements concerning the reproducibility, calibration and negative controls, as well as information about the evaluation of the pH transients are presented in four figures, one table and several equations. This material is available free of charge via the Internet at <http://pubs.acs.org>.

the planar surface of an ATR crystal modified with a thin gold layer to carry out time-resolved surface-enhanced IR absorption spectroscopy (tr-SEIRAS) triggered by flash-lamp excitation. The experiments suggest that both proteins interact in a cyclic fashion in both environments. The activity of the proteins seems to be preserved in the same way as in chromatophores or reconstituted in liposomes.

Graphical abstract

INTRODUCTION

Ubiquinol cytochrome c oxidoreductase, also referred to as the cytochrome bc_1 complex (bc_1), is located in the inner mitochondrial membrane of eukaryotes and the cytoplasmic membrane of prokaryotic organisms. The operation of bc_1 is usually discussed in terms of the Q-cycle first proposed by Peter Mitchell.¹ The first and rate limiting step in the Q-cycle is the oxidation of ubiquinol (UQH₂) in the Q_o site by the iron sulfur protein (ISP), which releases protons into the aqueous phase upon reaction with cytochrome c_1 . The semiquinone (SQ) formed at center Q_o is highly unstable² and transfers the second electron to the low potential chain of b hemes (b_L and b_H) to a quinone at center Q_i forming a SQ. A second turnover at the Q_o site leads to a second electron arriving at the Q_i site, reducing the SQ to UQH₂, thereby taking up protons from the opposite side of the membrane.^{3, 4}

Several modifications of the Q-cycle have since been discussed. For a reviews, see ^{4–6}.

According to this mechanism, the bc_1 contributes to the difference in electrochemical potentials of protons $\tilde{\mu}H^+$ across the lipid membrane that are used to drive ATP synthesis. $\tilde{\mu}H^+$ consists of the membrane potential, Φ , and a difference in pH values, ΔpH , between the inner and outer aqueous phases. A convenient way to study bc_1 is activation by the reaction centers (RCs) in the chromatophores of photosynthetic bacteria.^{4–8} Another important approach is the re-oxidation of bc_1 complexes reduced by ubiquinol using light-activated Ru complexes.^{9, 10}

The kinetics of electron transfer were thus investigated using UV/Vis spectroscopy, whereas pH and Φ transients that occur in chromatophores were observed using pH indicators, such as neutral red, and electrochromic band shifts of carotenoids or electrometric measurements, respectively.^{11–17} Although many details have been revealed, there are still a number of open questions to be solved about the exact mechanism of the bc_1 . This may be due to the

comparability of the results obtained by the two approaches mentioned above, because the solubilized enzyme cannot develop a $\tilde{\mu}H^+$, whereas proteins other than bc_1 are present in chromatophores that may interfere with the proteins under study. Moreover, the amount of ubiquinol is hard to control in both approaches. To overcome these drawbacks, we recently introduced proteo-lipobeads (PLBs), a biomimetic membrane system that is used for the oriented encapsulation of purified membrane proteins (MPs) in a functionally active form (Fig. 1A).^{18, 19}

PLBs are based on micrometer-sized agarose beads modified with Ni-NTA (nitrile tri-acetic acid)-terminated linkers (Fig. 1A). Membrane proteins (MPs) are bound to these linkers via histidine (his)-tags. When the particles are subjected to dialysis in the presence of lipid micelles, MPs are reconstituted into bilayer lipid membranes (BLMs) to form PLBs. The preparation technique was adopted from our previous studies on planar surfaces to form protein-tethered bilayer membranes (ptBLMs).²⁴ The format of PLBs offers the advantage of following pH and potential transients by fluorescence methods, i.e. confocal laser-scanning microscopy (LSM). In the present study, PLBs were employed to encapsulate RCs and bc_1 complexes from *R. sphaeroides* with the his-tag attached to the N side of the RC and the C-terminal end of the cytochrome (cyt) *b* subunit, so that the cyt *c* binding site of both proteins is directed to the outside of the PLBs. Hence, cyt *c* can be added to the surrounding solution, whereas ubiquinone can be co-reconstituted into the lipid bilayer in controlled quantities. RCs are then activated by illumination with a halogen lamp of an appropriate wavelength to activate the RC. The product of the illumination, UQH₂, will interact with the bc_1 complex but only in the presence of cyt *c*. As a result, protons are released into the outer aqueous phase. Fluorescence labels, such as fluorescein-DHPE incorporated into the lipid bilayer and the red-shifted potential sensitive dye di-4-ANBDQBS, are used to monitor the decrease of pH and generation of the membrane potential. As a consequence, information about the proton release kinetics of bc_1 is obtained under continuous illumination of the RC, controlled by the formation of the $\tilde{\mu}H^+$. The proton release kinetics are compared, as much as possible, with the flash-induced pH transients of bc_1 in chromatophores or reconstituted into liposomes.

To assess the turnover of bc_1 driven by the RC in a similar setting, we made use of the equivalent immobilization technique on planar surfaces (ptBLMs) mentioned above (Fig. 1B).²⁴ This technique allows flash-induced time-resolved surface-enhanced infrared absorption spectroscopy (tr-SEIRAS) to be applied to the two membrane proteins co-reconstituted into a lipid bilayer. (Fig. 1B)

The experiments suggest that both proteins cooperate in a cyclic fashion in both environments. From the results obtained so far, their activity seems to be preserved in the same way as in chromatophores or reconstituted in liposomes.

The purpose of this work is to present alternatives to existing methods of preparation and measurement of parameters such as turnover number and pH and potential transients to be used in further studies.

MATERIALS AND METHODS

Experiments on spherical particles

Rhodobacter sphaeroides RCs with a genetically engineered 7-his-tag at the C-terminus of the M-subunit were expressed from a strain kindly provided by S. G. Boxer.²⁵ RCs were purified according to a modification of the original method.²⁶

The *bc₁* complex poly-his-tagged on the C-terminus of the cyt *b* subunit was expressed and purified according to Guergova-Kuras et al.²⁷

The light harvesting complex apoprotein LHCP from *Pisum sativum* with a 6-his-tag attached to the C-terminus was expressed in *Escherichia coli*, strain JM101; reconstituted to functional LHCII monomers according to Paulsen et al.²⁸; and trimerized according to Rogl et al.²⁹ The pigments were extracted from *Pisum sativum* according to Paulsen et al.³⁰

The compound 4-(1-[2-(di-*n*-butylamino)-6-naphthyl]-4-butadienyl)-1-(4-butylsulfonate) quinolinium betaine (di-4-ANBDQBS) was synthesized according to Matiukas et al.³¹

Agarose beads (Thermo Scientific HisPur Ni-NTA Resin (PI-88221), 50–150 µm) were purchased from Fisher Scientific (Waltham, Massachusetts, USA). 1,2-Diphytanoyl-*sn*-glycero-3-phosphocholine (DiPhyPC, > 99%) was purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). Fluorescein-DHPE (1-(8-((3',6'-dihydroxy-3-oxo-spiro(isobenzofuran-1(3H), 9'-(9H)xanthen)-5-yl)amino)-3-hydroxy-8-thioxo-2,4-dioxo-7-aza-3-phosphaoct-1-yl)-1,2-ethanediyl ester, P-oxide) was purchased from Invitrogen (Waltham, Massachusetts, USA). Potassium chloride, tris(hydroxymethyl)aminomethane (Tris-HCl), *n*-dodecyl β-D-maltoside (DDM), chloroform, dimethyl sulfoxide (DMSO), valinomycin (1 mg/mL in DMSO, 0.2 µm filtered), carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP, 98%), cytochrome *c* from bovine heart, imidazole and ubiquinone-10 (Q-10, 2,3-dimethoxy-5-methyl-6-all-*trans*-decaprenyl-1,4-benzoquinone) were purchased from Sigma-Aldrich (Steinheim, Germany). Float-A-Lyzer (MWCO: 500–1000 Da, volume 5 mL) dialysis tubes and poly(methyl methacrylate) cuvettes with 10 mm light path were obtained from Carl Roth (Karlsruhe, Germany). Flow cells (µ-slide I 0.6 Luer) were purchased from ibidi. PD-10 Sephadex[®] gel filtration columns (G-25M) were obtained from GE Healthcare (Little Chalfont, UK).

Preparation of proteobeads—Agarose bead slurry (500 µL, HisPur Ni-NTA Resin) was repeatedly rinsed, centrifuged (Heraeus Fresco Microcentrifuge, Thermo Scientific, Waltham, Massachusetts, USA) for 1 min at 1.5×10^3 g and re-suspended, first in a Tris-HCl/KCl buffer solution (5 mM/35 mM) three times and then three times in a DDM Tris-HCl/KCl buffer solution (5 mM Tris HCl, 35 mM KCl, pH = 8, 0.1% DDM (*n*-dodecyl β-D-maltoside)). After washing, the remaining 250 µL of pellets was re-suspended in 1 mL of DDM Tris-HCl/KCl buffer, and 50 µL of a 10 µM stock solution of RC and *bc₁* complexes each were added, corresponding to 5×10^{-10} mol. After 2 h of incubation with gentle pivoting, the beads were rinsed again, centrifuged and re-suspended three times in DDM Tris-HCl/KCl buffer to remove unbound and nonspecifically adsorbed proteins.

To determine the concentration of the proteins bound to the proteobeads, the sample was centrifuged and the pellet was re-suspended in 425 μL of a DDM Tris-HCl/KCl buffer solution containing 300 mM of imidazole. After 1 h of incubation with gentle pivoting, the sample was centrifuged again. The supernatant was collected and the procedure was repeated one additional time. The concentration of the RC and *bc*₁ were determined by UV/Vis spectroscopy (see below) in the collected supernatants. The quantities recovered after imidazole treatment were 4.3×10^{-10} and 4.6×10^{-10} mol, respectively, corresponding to 85.7 and 91.7 %, respectively of the amount originally added to 250 μL of bead pellets, which was used to calculate the number of protein complexes per sample for the measurements. The spectra are shown in Fig. S1.

Preparation of the proteo-lipobeads (PLBs)—A Spectra/Por Float-A-Lyzer (MWCO: 500–1000 Da, volume 5 mL) dialysis tube was filled with the collected proteobead suspension, and 3 mL of a lipid suspension containing 40 μM DiPhyPC and 6 μM ubiquinone Q-10, dissolved in DDM Tris-HCl/KCl buffer, was added. Lipids and Q-10 were first dissolved in chloroform, which was then evaporated, followed by 2 hours of swelling in the aforementioned buffer at 30 – 40 °C, rapid vortexing for 10 min and incubation overnight at 4 °C. The sample was dialyzed in 1 L of Tris-HCl/KCl buffer at room temperature for 24 hours with 3 complete dialysate changes after 2, 4 and 10–14 hours. The PLB suspension was allowed to sediment, and 40 μL of the deposit was used for the experiments. The 40- μL deposit corresponds to 12.3 μL of the PLB pellet after centrifugation. The amount of pellet was determined by weighing the supernatant.

Labeling with potential-sensitive fluorescent dye—A stock solution of di-4-ANBDQBS in ethanol (20 μL , 1 mg/5 mL) and 40 μL of PLB deposit was brought up to 1 mL with Tris-HCl/KCl buffer to obtain a final concentration of 6.9 μM di-4-ANBDQBS. After incubation for 20 min, the PLBs were washed three times in dye-free Tris-HCl/KCl buffer by rinsing, centrifugation and re-suspension.

Labeling with pH-sensitive fluorescent dye—The stock solution of fluorescein-DHPE in chloroform (20 μL , 1 mg/5 mL) was added to a suspension of 40 μL of PLB deposit in 940 μL of Tris-HCl/KCl buffer to a final concentration of 3.3 μM fluorescein-DHPE. After incubation for 20 min in fluorescein-labeled DHPE, the PLBs were separated from chloroform, and then, the PLBs were washed three times in dye-free Tris-HCl/KCl buffer by rinsing, centrifugation and re-suspension.

The characteristics of the fluorescent dyes are shown in Table 1.

Preparation of reduced cyt *c*—Horse heart cyt *c* (25–30 mg) was dissolved in a Tris-HCl/KCl buffer solution. Cyt *c* reduction was achieved by admixing 0.3 mg of sodium hydrosulfite. A PD-10 Sephadex[®] gel filtration column was used to separate the excess hydrosulfite from the reduced cyt *c*. The concentration of the collected cyt *c* solution was determined by UV/Vis spectroscopy (see below). Cyt *c* was added to the sample to a final concentration of 0.1 mM.

UV/Vis spectroscopy—UV/Vis spectra were measured on a Hitachi U-2900 spectrophotometer (Hitachi, Ltd., Tokyo, Japan) using poly(methyl methacrylate) cuvettes with a 10-mm light path obtained from Carl Roth. For the determination of the amount of protein bound to the proteobeads, UV/Vis spectra of proteins detached from Ni-NTA-functionalized agarose beads were measured using $\epsilon_{562-575\text{ nm}}^{\text{reduced-oxidized}} = 25.6\text{ mM}^{-1}\text{cm}^{-1}$ (dithionite, ferricyanide) for the bc_1 complex³² and $\epsilon_{802} = 288\text{ mM}^{-1}\text{cm}^{-1}$ for the RC.³³ For the determination of the concentration of reduced cyt *c*, UV/Vis spectra were measured using $\epsilon_{550} = 27.7\text{ mM}^{-1}\text{cm}^{-1}$, according to Margoliash and Frohwirt.³⁴ Spectra were evaluated with OriginLab 8.5 (OriginLab Corporation, Northampton, Massachusetts, USA).

Confocal Laser-Scanning Fluorescence Microscopy (LSM)—Confocal laser-scanning fluorescence microscopy measurements were carried out on an upright Leica TCS SP5 II microscope with a 10x dry objective (Leica, HC PL APO 10x/0.40 CS, Leica Microsystems, Wetzlar, Germany). The labeled PLBs were re-suspended in Tris-HCl/KCl buffer, 20 μL of a 200 μM stock solution of both cyt_{red} and cyt_{ox} was added and brought up to 400 μL . The collected sample was transferred to a flow cell (μ -slide upright, ibidi GmbH, Munich, Germany). The 561 nm line of a diode pumped solid state laser was used for the excitation of di-4-ANBDQBS. Fluorescence was detected at 685–790 nm. For fluorescein-DHPE-labeled PLBs, the 488 nm line of a multi-line argon laser was used for excitation, and fluorescence emission was detected at 500–600 nm (Table 1). Images were taken every 1.3 s before, during and after 60 s of continuous illumination with a halogen lamp (Fiber-lite DC 950, Dolan Jenner Industries). These images were then used to analyze localized intensities in the immediate vicinity of the PLBs using the software Leica Application Suite Advanced Fluorescence (Leica Microsystems, Wetzlar, Germany). Further evaluation was performed with OriginLab 8.5 (OriginLab Corporation, Northampton, Massachusetts, USA).

Experiments on planar surfaces

3-Mercaptopropyltrimethoxysilane (MPTES, 95%) was purchased from ABCR GmbH (Karlsruhe, Germany). Gold granules (99.99%) for evaporation were obtained from Mateck GmbH (Juelich, Germany). Bio-beads (20–50 mesh) were purchased from Bio-Rad Laboratories GmbH (Vienna, Austria). 1,2-Diphytanoyl-*sn*-glycero-3-phosphocholine (DiPhyPC, >99%) was provided by Avanti Polar Lipids (Alabaster, Alabama, USA). Dithiobis (nitriloacetic acid butylamidyl propionate) (DTNTA, 95.0%) was obtained from Dojindo Laboratories (Kumamoto, Japan). Hydroxylamine hydrochloride ($\text{NH}_2\text{OH}\cdot\text{HCl}$, 99%), gold(III) chloride hydrate ($\text{HAuCl}_4\cdot x\text{H}_2\text{O}$, 99.999%), dimethyl sulfoxide (DMSO, puriss., dried over molecular sieve), 3,3'-dithiodipropionic acid (DTP, 99%), dodecyl- β -D-maltoside (DDM, 98%), nickel(II) chloride (NiCl_2 , 98%), D-(+)-glucose ($\text{C}_6\text{H}_{12}\text{O}_6$, 99.5%), glucose oxidase (GOX) and catalase, as well as ubiquinone-10 (Q-10, 2,3-dimethoxy-5-methyl-6-all-*trans*-decaprenyl-1,4-benzoquinone) were purchased from Sigma-Aldrich (Steinheim, Germany). All chemicals were used as purchased.

Preparation of the two-layer gold surface on the ATR crystal—The preparation was performed as previously described.³⁵ A polished silicon attenuated total reflection (ATR) crystal was immersed in a 10 % ethanolic solution of MPTES for 60 minutes to anchor the gold layer. After rinsing with ethanol, the sample was dried under a stream of

argon and annealed at 100 °C for 60 minutes. After cooling to room temperature, the crystal was immersed in water for 10 minutes and dried under a stream of argon. A 25 nm gold film was then deposited onto the ATR crystal by electrochemical evaporation (HHV Edwards Auto 306, Crawley, UK). Gold nanoparticles were grown on the gold film by immersing the crystal in 50 mL of an aqueous solution of hydroxylamine hydrochloride (0.4 mM), to which 500 μ L of an aqueous solution of gold(III) chloride hydrate (0.3 mM) was added five times at 2-minute intervals. Finally, the sample was rinsed with water and dried under a stream of argon.

Immobilization of the protein—*Rhodobacter sphaeroides* RCs with a genetically engineered 7-his-tag at the C-terminus of the M-subunit and the bc_1 complex poly-his-tagged on the C-terminus of the cyt *b* subunit were immobilized on the two-layer gold surface on top of the ATR crystal, prepared according to a method described earlier³⁶, as well as references therein. Briefly, the gold surface was immersed in a solution of 2.5 mM DTNTA and 7.5 mM DTP in dry DMSO for 20 h. After rinsing with ethanol and purified water, the surface was immersed in 40 mM NiCl_2 in acetate buffer (50 mM, pH 5.5) for 30 minutes, followed by thorough rinsing with purified water to remove excess NiCl_2 . The surface was dried under a stream of argon prior to assembly in the measuring cell and rehydrated with DDM phosphate buffer (DDM-DPK) (0.05 M K_2HPO_4 , 0.1 M KCl, pH = 8, 0.1% DDM). RCs and bc_1 complexes dissolved in DDM-DPK were adsorbed to the NTA-functionalized gold surface, both at a final concentration of 100 nM. After a 4 h adsorption time at 28 °C, the cell was rinsed with DDM-DPK to remove nonspecifically adsorbed and bulk protein. Thereafter, DDM-DPK was replaced by a DiPhyPC/DDM-DPK solution (40 μ M DiPhyPC in DDM-DPK). In the case of additional ubiquinone, Q-10 was solubilized together with DiPhyPC (6 μ M Q-10 in DiPhyPC/DDM-DPK). After incubation, DDM was removed by adding Bio-beads to the lipid-detergent solution. Finally, cyt *c* was added to the buffer solution to a final concentration of 0.1 mM.

Attenuated total reflection surface-enhanced infrared absorption

spectroscopy (ATR-SEIRAS)—SEIRA spectroscopy was performed in a flow cell that was originally designed for the electrochemical excitation of the protein and was mounted on top of a trapezoid single reflection silicon ATR crystal. The IR beam of the FTIR spectrometer (VERTEX 70v, Bruker, Ettlingen, Germany) was coupled into the crystal at an angle of incidence $\Theta = 60^\circ$ by using the custom-made setup described previously.³⁵ All spectra were measured with parallel polarized light. Because the ATR element surface is coated with an electrical conductor, perpendicularly polarized light is unable to penetrate the conducting layer effectively. The total reflected IR beam intensity was measured with a liquid nitrogen-cooled photovoltaic mercury cadmium telluride (MCT) detector.

Thereafter, IR measurements were performed at 28 °C. The sample chamber housing the flow cell was purged with dry carbon dioxide-free air to remove CO_2 and water vapor from the light path. FTIR spectra were recorded at a 4 cm^{-1} resolution using Blackham-Harris 3-term apodization and a zero filling factor of 2. The interferograms were measured in the double-sided mode and transformed into spectra using the power mode of Fourier transformation. Sample illumination was provided by a L11316-12 XENON flash lamp

module provided with an A7969-08AS lightguide and A11690 connector obtained from Hamamatsu. Spectra were analyzed using the software package OPUS 7 (Bruker, Ettlingen, Germany) and OriginLab 8.5 (OriginLab Corporation, Northampton, Massachusetts, USA).

RESULTS AND DISCUSSION

Experiments on spherical particles

A 1:1 mixture of solubilized RCs and *bc*₁ complexes was bound to the agarose beads to form proteobeads. The amount of protein bound to 250 μ L of agarose bead pellets was determined to be 4.1×10^{-10} mol and 4.6×10^{-10} mol, respectively, corresponding to 85.7 and 91.7 % RCs and *bc*₁ complexes, respectively, of the amount originally added, what is in good agreement with the amount of protein bound to the PLBs reported previously by Schadauer et al.¹⁹ UV/Vis spectra are shown in Fig. S1.

After addition of the DiPhyPC/Q-10 containing lipid solution, the proteobeads were then subjected to dialysis, as described, to form PLBs. The formation of the lipid bilayer membrane (BLM) was confirmed by LSM using an appropriate fluorescent label. The lipid bilayer was labeled with fluorescein-DHPE, and fluorescence emission was measured at 500 – 600 nm. Figure 2 illustrates a layer of regular fluorescence intensity around the bead. In this approach, PLBs were prepared from light harvesting complex II (LHCII) of green plants, also provided with a his-tag, which also has a fluorescence emission (at 650 – 700 nm). Both of these images are superimposable, showing that proteins and lipids are, in fact, located in the same layer surrounding the beads (Fig. 2). The sealing properties are discussed in the following sections.

pH and potential transients measured by fluorescence microscopy—RCs and *bc*₁ complexes were co-reconstituted via his-tags attached to the N side of the RCs and the C-terminal end of the cyt *b* subunit, respectively. PLBs were thus obtained presenting the cyt *c* binding site of both proteins to the outside of the PLBs (Fig. 1C). This orientation is opposite to the orientation found in chromatophores; however, this is the proper orientation of the proteins relative to each other. Hence, this arrangement favors their natural interaction, particularly when ubiquinone-10 (UQ) was co-reconstituted in controlled quantities and cyt *c* was added to the aqueous phase. The required amount of UQ (see the experimental section) was derived from previous studies of RCs in a similar setting. Under continuous illumination with a halogen lamp, FTIR indicated the formation of the ubihydroquinone of UQ, UQH₂, as well as the oxidized form of the special pair, P870⁺.³⁶ UQH₂ is needed as the donor of electrons and protons to the *bc*₁ complex, whereas cyt *c* is required to accept electrons from *bc*₁ complexes, thus facilitating the release of protons. Reduced cyt *c*, in turn, is passed to the RC to reduce P870⁺ to the ground state, P870, thus allowing the two proteins to interact continuously in a circular fashion (Fig. 1C).^{5, 6} Control experiments without cyt *c* (Fig. 3) demonstrated the essential role of this protein. Under these conditions, RCs were repeatedly activated with a halogen lamp. The pH changes in the outer aqueous solution of the PLBs were detected by fluorescein-DHPE (Table 1), a sensor molecule that incorporates into the distal leaflet of the lipid bilayer. The decrease in pH was detected by a decrease of fluorescence emission intensity. LSM images and fluorescence emission intensities were

recorded within localized areas before and after illumination. The results are shown in Fig. 3.

The pH decreased on a time scale of seconds when the halogen lamp light was switched on and remained on the level attained during illumination when the halogen lamp was switched off. The experiments were repeated 4 times and were reproducible within a margin of 7.2 % of the final value (Fig. S3). The fluorescence emission intensity was calibrated vs. pH by immersion of the labeled PLBs into KCl/citrate solutions (35/5 mM) titrated to pH values ranging from pH = 3 to 10. For the calibration plot, see Fig. S2.

The pH transients were evaluated by comparing the relative fluorescence intensities, using the calibration function of fluorescein-DHPE given in the supplementary information (Fig. S2). As a result, a decrease of pH from pH = 8.0 to approximately pH = 7.2 was obtained at the end of 7 illumination steps. Data from the first illumination step were fitted to a mono-exponential function of pH vs. time (Fig. 3B). Using continuous illumination, we consider the protein complexes to be activated one by one rather than simultaneously as in the case of flash excitation. Therefore, we report only the initial rates per protein at the onset of the continuous illumination, when only the first proteins undergo their reactions. Taking into account the buffer capacity of the Tris buffer, as well as the number of protein complexes according to 85.7 % and 91.7 % of the amount added to the agarose beads, the initial proton release rates per *bc₁* complex were obtained. Bleaching effects, determined in the course of the control experiments without RC and cyt *c*, respectively, were subtracted. The buffer capacity of the fluorescein-DHPE was found to be negligible. Details of the calculation are presented in the supplementary information.

As a result, initial proton release rates of 286 and 663 H⁺ s⁻¹ per *bc₁* complex were obtained in the absence and presence of valinomycin, respectively. The ratio between the two rates amounts to 2.3 reflecting the difference between the coupled and uncoupled enzyme. A ratio higher than 2 is generally considered to indicate good sealing properties of the lipid bilayer.³⁶ These results, however, cannot be directly compared with flash-induced pH transients measured on chromatophores, because we used continuous light for the excitation. Anyhow, flash-induced pH transients within the lumen of chromatophores of *Rhodobacter capsulatus* were obtained, whereas potential transients were monitored by the electrochromic band shift of carotenoids.^{37, 38} As a result, the rise of the electrochromic transient exactly followed the slow phase of the luminal acidification with a half rise time of approximately 10 ms, which corresponds to 100 s⁻¹.

To monitor changes of the membrane potential during the circular interaction of the two proteins, PLBs labeled with di-4-ANBDQBS were subjected to illumination with a halogen lamp. The fluorescence intensity decreased, i.e., the membrane potential (MP) that was positive outside first decreased on the time scale of seconds and then changed in the reverse direction when the light was still switched on and increased only slightly further when the light was switched off.

Correlating the two sets of measurements (Fig. 4), we can deduce that protons are released immediately at the onset of illumination, while the MP decreases in the negative direction,

but only in the first half of the illumination phase. Subsequently, in the second half and further when the light is switched off, the initial decrease is reversed by the generation of the MP (positive outside) through the bc_1 complex. For an explanation, we have to consider that in our setup both proteins generate a potential of the same polarity after activation, i.e., the outside is positive. However, the contribution from the RC is expected to vanish as the products of illumination, $P870^+$ and UQH_2 , are consumed in reaction with bc_1 complexes. This assumption is in accordance with the decrease of fluorescent intensity of di-4-ANBDQBS in the first phase of illumination, particularly because the turnover of bc_1 is slow compared to the RC. Therefore, the initial stage of proton transfer is limited by the positive potential generated by the RC. This potential reaches a minimum when bc_1 starts to compete kinetically with the RC. Thereafter, the potential positive outside generated by bc_1 will reverse the direction. We conclude that the Φ builds up across the membrane as protons are released by bc_1 , accounting for proton transfer eventually reaching saturation. This assumption seems justified in the light of a control experiment performed in the presence of valinomycin and FCCP, showing the absence of the potential increase due to the bc_1 (Fig. S4).

Experiments on planar surfaces

Time-resolved surface-enhanced IR absorption spectroscopy (tr-SEIRAS) of the bc_1 co-reconstituted with the RC in a ptBLM—Co-immobilization of RC and the bc_1 complex via his-tags attached to the P side of the RC and the C-terminus of the cyt b subunit was achieved on a planar surface, optimized with respect to the enhancement effect of nano-structured gold surfaces.³⁵ Immobilization was indicated by an increase of vibrational components in the amide I region, with a maximum at 1652 cm^{-1} , similar to that obtained earlier with the RC alone.³⁶ Discrimination from the O-H bending mode of H_2O (1642 cm^{-1}) was achieved by a calculation of the ratio of the H_2O stretching and bending modes as a function of the time of the immobilization. A variation of the ratio in the course of the adsorption indicates that a species other than H_2O had been adsorbed to the surface. Thus, we made sure that the vibrational components of approximately 1652 cm^{-1} were indeed dominated by proteins, which were then subjected to in-situ dialysis, as described in the experimental part as well as in our previous publications.^{36, 39, 40}

The flow cell was provided with the fiber optic of the flash lamp, and tr-SEIRA spectra were recorded using the step-scan technique triggered by the flash lamp at frequencies of 50 and 100 Hz. Tr-spectra after flash light excitation at a frequency of 100 Hz are shown in Fig. 5.

The spectra show changes of approximately 1290 , 1380 , 1440 , 1655 and 1730 cm^{-1} . Similar spectra were also obtained at a frequency of 50 Hz. Orthogonal phase-resolved spectra were prepared for the exact location of the bands for both frequencies at 1298 , 1387 , 1451 and 1654 cm^{-1} (Fig. 6).

The bands between 1200 and 1700 cm^{-1} correspond to those obtained by the SEIRA spectra that were measured before of the RC alone reconstituted into the same ptBLM structure.³⁶

Significant changes, however unspecific, are also observed in the region of the amide I bands, at approximately 1650 cm^{-1} . They are tentatively assigned to conformational changes

in the bc_1 complex, e.g., due to the mobility of the Iron Sulfur Protein changing positions¹⁰ between that close to b -hemes to that close to cytochrome c_I during turnover.

Band changes were observed in our earlier work regarding the RC alone, as mentioned above, albeit under continuous illumination, appearing at 1282, 1360, 1434, and 1642 cm^{-1} . The small number of bands was surprising because the steady state FTIR difference spectra of the RC after continuous illumination found in the literature are usually composed of a large number of narrow peaks and troughs, e.g.,⁴¹.

The unusually small number of absorption bands detected after light excitation is a consequence of both the modulated excitation and the gold layer covering the surface of the ATR crystal. Modulated excitation in combination with phase-resolved or difference spectra reduces the absorbance spectrum to bands that change as a consequence of light excitation and rejects all other bands that are not involved in the chemical processes that take place during the experiment. However, the gold layer reduces the detectable absorption to transition dipole moments with components on the z -axis, i.e., perpendicular to the membrane layer, resulting in stronger absorption for transition dipole moments of the respective functional groups that are oriented perpendicular to the surface.^{42, 43}

Therefore, the same component present in different orientations may be strongly represented or not at all. This effect is still more enhanced by the pre-orientation of protein molecules due to his-tag binding. Based on the FTIR spectra published in the literature, the very strong bands at 1434 and 1282 cm^{-1} are attributed to the OH stretching vibration of UQH_2 and the complex band of the excited state P870^+ of the RC, respectively. In the present work, we concentrated on the region between 1500 and 1400 cm^{-1} , which we consider to be mainly composed of the 1434 cm^{-1} band. This band is expected to change in a periodic fashion when UQH_2 , produced by the RC, is consumed by the bc_1 complex. The fact that UQH_2 seems to be present on the surface in an oriented fashion can be explained in terms of the Q_B site of the RC. UQH_2 is known to be fixed locally within the Q_B site in a specific orientation. To be sure that spectral changes do occur at approximately 1440 cm^{-1} , the spectra were subjected to a treatment called phase sensitive detection (PSD). PSD revealed significant changes in this particular spectral range (Fig. 6). Consequently, absorbances were integrated from 1400 to 1500 cm^{-1} and plotted vs. time, resulting in the time-resolved absorbance change depicted in Fig. 7.

The signal can be seen to change in a periodic fashion, as expected for a substrate of the bc_1 complex. The decrease of the bands at approximately 1440 cm^{-1} was fitted to a mono-exponential function. With a flash lamp used for excitation, the protein complexes are considered to be activated simultaneously. Therefore, we can directly derive the time constant to be $\tau = 3.17$ ms, which corresponds to a turnover number of the bc_1 complex embedded into a ptBLM of 316 $\text{e}^- \text{s}^{-1}$. This value can be considered to be the actual turnover number because UQH_2 is the substrate that donates electrons to the enzyme cycle.

CONCLUSIONS

The protein pair RC and bc_1 embedded in the ptBLM structure both on spherical and flat surfaces appears to cooperate repeatedly in a circular fashion when the RC is illuminated by white light. The reaction reaches saturation, presumably limited by the generation of the $\tilde{\mu}H^+$. The reaction can be repeated several times within very narrow margins. We conclude that the proteins retain their activity. To do so, substrates of the proteins, such as UQ and cyt c_{red} of RC and UQH₂ and cyt c_{ox} of bc_1 , must be formed and consumed repeatedly. Hence, the concentration of components added, UQ-10, cyt c_{red} and cyt c_{ox} , seem not to be the limiting factor.

The actual turnover of bc_1 therefore is accessible on the planar surface of an ATR crystal (Fig. 1B). The proteins are embedded into a ptBLM of the same orientation as that employed for the PLB. The RC is initiated by single light flashes, and tr-SEIRAS is performed at an appropriate frequency. The step scan technique did not allow for a time resolution low enough to detect single steps of the enzyme cycle. The step scan technique demonstrates, however, periodic changes of UQH₂ as the substrate of the bc_1 complex. The time constant obtained ($\tau = 3.17$ ms) can be compared with the turnover number of the purified bc_1 complex, which was reported to be $v_{max} = 333 \text{ s}^{-1}$.¹¹ This number reflects the turnover of electrons rather than protons. The overall reaction of the bc_1 according to Crofts¹² is described by:

For every turnover of bc_1 consuming two electrons, four protons are released to the positive side of the membrane. Hence, the proton release rate is expected to be twice the turnover number, at least for an uncoupled enzyme.

Time constants could not be obtained from measurements with PLBs because we had to use continuous light for activation. Therefore, we use the initial slope of the pH transient to derive the initial rates of proton release per bc_1 when the bc_1 is still in the initial stage of turnover. Anyhow, flash-induced pH transients within the lumen of chromatophores of *Rhodobacter capsulatus* showed a half rise time of approximately 10 ms. This corresponds to a proton release rate of 100 s^{-1} .

We conclude that the initial proton release rates obtained from our measurements are on the order of magnitude of time constants obtained from flash-induced illumination of chromatophores.

This result is unexpected considering our earlier studies using PLBs based on silica nanoparticles with a diameter of 25 to 50 nm. Using UV/Vis spectroscopy, the turnover of cytochrome c oxidase (CcO) from *P. denitrificans* was found to be orders of magnitude smaller than 500 s^{-1} , the value accepted for the uncoupled enzyme.²⁰ We argued that the reduced turnover was due to the binding of CcO via a his-tag. Because of the present results, this argument has to be reconsidered. We consider the inner volume of the PLBs to play a larger role compared to binding. The length of the spacer and lipid bilayer including proteins

of 2 and 10 nm, respectively, is negligible vs. the average size of the agarose-based PLBs, which are 100 μm diameter. Hence, almost the entire volume of the PLB equals its inner volume underneath the lipid bilayer. As agarose has, in contrast to the silica particles used in the above-mentioned study, a considerable storage capacity for water molecules and ions, such as protons, an extensive reservoir for protons is available to the protein. We conclude that the reservoir of agarose-based PLBs is not a limiting factor of proton transfer across the membrane. This explains why the proton release rate is close to the kinetic data of the bc_1 complex in chromatophores.

In summary, we conclude that PLBs as well as the ptBLM on spherical and planar surfaces, respectively, can be used as an experimental model for the cell-free investigation of purified membrane proteins. The main advantage of this model over other model systems, such as liposomes, is the strict control over the orientation, an important parameter as far as directed transport is concerned. Another benefit is the relatively large storage capacity of the inner volume of PLBs compared to cell organelles, such as mitochondria and chromatophores.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

PLB	proteo-lipobead
<i>bc</i>₁	ubiquinol cytochrome <i>c</i> oxidoreductase/cytochrome <i>bc</i> ₁ complex
RC	reaction center
cyt <i>c</i>	cytochrome <i>c</i>
ptBLM	protein-tethered bilayer lipid membrane
LSM	confocal laser-scanning fluorescence microscopy
ATR-SEIRAS	attenuated total reflection surface-enhanced infrared absorption spectroscopy
tr-SEIRAS	time-resolved surface-enhanced infrared absorption spectroscopy

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Figure 1.

RC and bc_1 proteins immobilized on the spherical surface of an NTA-modified agarose bead (turquoise) (A) and the planar surface (gold) of an ATR crystal (transparent blue) (B). The cytochrome c binding site of both proteins is located on the outer side of the lipid layer. *Rhodobacter sphaeroides* RCs (green; H subunit: yellow-green, L subunit: medium green, M subunit: blue-green; PDB file: 2UXJ²⁰) with a genetically engineered 7-his-tag (dark green) at the C-terminus of the M subunit, and bc_1 complexes (orange; A and D subunit: medium orange, B and E subunit: yellow-orange; C and F subunit: red-orange; homodimer, so A is equivalent to D, B to E, and C to F; PDB file: 2QJK²¹) poly-his-tagged (dark orange) on the C-terminus of the cyt b (A, D) subunit immobilized (linker in gray) within a lipid bilayer (white; PDB file: dppc64²²). Cytochrome c is depicted in red (PDB file: 2B4Z²³). (C) Detailed membrane scheme naming the most important components of the employed system. Components are colored as above except from the lipid bilayer and the linker molecule (by element). In addition, ubiquinone Q-10 (UQ) is depicted in magenta, fluorescein chromophores in transparent green, water molecules in blue and protons in yellow. Fluxes are schematic, chemical equivalency is not given.

Figure 2. LHCII-PLBs labeled with fluorescein-DHPE. (A) Channel 1: $\lambda_{\text{ex}}/\lambda_{\text{em}}$ 488/500 – 600 nm (fluorescein-DHPE emission). (B) Channel 2: $\lambda_{\text{ex}}/\lambda_{\text{em}}$ 488/650 – 700 nm (LHCII emission). (C) Superposition of Channel 1 and 2.

Figure 3.

(A) Fluorescence intensity as a function of time measured at RC and bc_1 complexes co-reconstituted with Q-10 within PLBs labeled with fluorescein-DHPE, with (black, blue and green) and without cyt *c* (red) added to the aqueous solution. Samples were illuminated with the halogen lamp. Control experiments were performed without cyt *c* (red), with bc_1 alone (blue) and in the presence of valinomycin (green). Arrows indicate when the halogen lamp is switched on (↑) and off (↓). (B) Evaluation of the first illumination step. pH values obtained from the calibration plot (Fig. S2) vs. time are fitted to a mono-exponential function (see the supplementary information for details). The red lines show the fitted curves.

Figure 4.

RC and bc_1 complexes co-reconstituted in PLBs with Q-10, cyt *c* added to the aqueous solution. Correlation of the two sets of measurements: pH and potential transients measured with fluorescein-DHPE and di-4-ANBDQBS labels, respectively. Arrows indicate when the light is switched on ($\uparrow$) and off ($\downarrow$).

Figure 5.

tr-SEIRA spectra of the RC and bc_1 complexes embedded into a ptBLM excited by illumination with a flash lamp at a frequency of 100 Hz. The sequence of the spectra is color coded: blue (1–5), green (6–10), pink (11–15), light blue (16–20), red (21–25). The mean of all of the intensity spectra measured was used as the reference for the calculation of the time-resolved absorbance spectra.

Figure 6. Overall absorbance changes during the modulated light-excitation calculated as the amplitude of two orthogonal phase-resolved spectra (mean value of two experiments for each frequency). Excitation frequencies: 50 Hz (red) and 100 Hz (blue). Shaded areas indicate the integrated areas used for data evaluation of Fig. 7.

Fig. 7.

Time-resolved absorbance change as a function of time obtained from the time-resolved SEIRA spectra shown in Fig. 6. The integrated spectral range was 1400–1500 cm⁻¹. The decreasing branch of the data was fitted to a mono-exponential function (red), from which the time constant was derived to be 3.17 ms.