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Interactions of Photosynthetic Reaction Centers with Bc_1 Complexes from *Rhodobacter Sphaeroides* Studied Using SEIRAS on a Nano-Structured Gold Surface

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Abstract: Inter-protein reactions of photosynthetic reaction centers (RCs) with bc_1 complexes from *R. sphaeroides*, have been investigated using surface-enhanced Infrared-absorption spectroscopy (SEIRAS). Surface enhancement was achieved by a nano-structured gold surface. The proteins were immobilized via his-tags attached to the P side of the RC and the C-terminal end of the cytochrome (cyt) *b* subunit and co-reconstituted into a lipid bilayer by *in-situ* dialysis. In this configuration, the cyt *c* binding site of the two proteins is located on opposite sides of the membrane. Light-minus-dark absorbance spectra under continuous illumination in the absence of an electron donor indicated a slow quinone/semiquinone exchange, allowing release of ubiquinol (QH_2) into the membrane. The interaction of the bc_1 with QH_2 was indicated by the stationary state obtained but only in the presence of cyt *c*. The interaction is discussed in terms of a semiquinone species formed in the course of the Q cycle mechanism of the bc_1.

Keywords: Photosynthetic Reaction Centers, Bc_1 Complexes, *R. Sphaeroides*, Surface-Enhanced Infrared-Absorption Spectroscopy, Nano-Structured Gold Surface, Ubiquinol, Semiquinone, His-Tags

1. Introduction

The investigation of membrane proteins is facilitated by the immobilization on a surface via his-tag technology. Monolayers of proteins thus obtained can be reconstituted by *in-situ* dialysis into protein-tethered bilayer lipid membranes (ptBLMs) [1]. The proteins can thus be investigated in a functionally active form using SEIRAS (surface-enhanced infrared spectroscopy) [2] and SERRS (surface-enhanced resonance Raman spectroscopy) [3]. SEIRAS in the ATR mode has been successfully applied to several membrane proteins such as cytochrome *c* oxidase [4-7]. Rhodopsin [8] and the reaction center (RC) of photosynthetic bacteria [9], in a ptBLM configuration. Surface-enhancement is achieved by nano-structured gold or silver surfaces. Here, we report on the co-reconstitution of RCs with the bc_1 complex from *R. sphaeroides* via his-tags attached to the P side of the RC and the C-terminal end of the cytochrome (cyt) *b* subunit (Fig. 1).

The interaction of the bc_1 complex with the RC has been demonstrated before to interact via ubiquinol and an electron carrier cyt *c* through flash-induced electron transport in *R. sphaeroides* chromatophores [10]. Reverse electron transfer within the bc_1 complex can be shown by the electric field generated by the RCs [11]. FTIR spectra of the RC and the

bc_1 complex isolated from bacteria have been widely reported, mostly in the form of reduced-minus-oxidized difference spectra, e.g. [12-16]. We have previously shown that SEIRAS allows to reveal slow inter-protein reactions between RC molecules, once the RC has been activated by continuous illumination with a quartz halogen lamp [9]. In the present study, we explore the same inter-protein reactions of RCs in the presence of the bc_1 complex. Even though the cyt *c* binding site is located on opposite sides of the proteins, the turnover of the bc_1 complex has been demonstrated elicited by cyt *c* in the aqueous phase. As a requirement for this process a certain mobility of semiquinone species is considered.

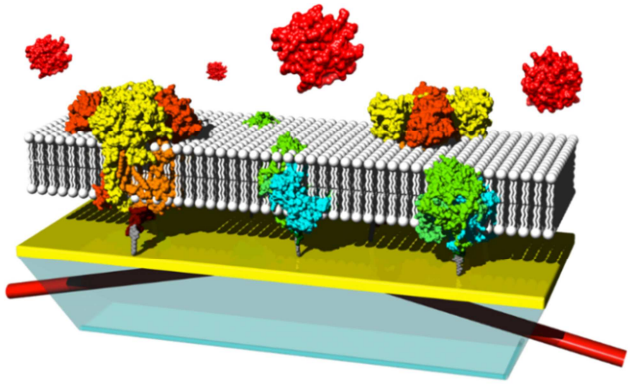

Figure 1. *RCs and bc_1 complexes immobilized on a NTA-functionalized gold surface, co-reconstituted in the ptBLM. RCs are immobilized with the primary donor (P) and the bc_1 complexes with the C-terminal end of the cyt b subunit oriented towards the surface of a silicon ATR crystal, covered with a nano-structured gold film.*

2. Experimental Procedures

2.1. Solvents and Chemicals

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, AL). Dithiobis (nitriloacetic acid butylamidyl propionate) (DTNTA, ≥95.0%) was obtained from Dojindo Laboratories (Kumamoto, Japan). Hydroxylamine hydrochloride ($NH_2OH.HCl$, 99%), gold(III) chloride hydrate ($HAuCl_4.xH_2O$, 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 ($C_6H_{12}O_6$, ≥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.

2.2. Preparation of the Two-Layer Gold Surface on the ATR Crystal

Preparation was done as previously described [17]. 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 µL of an aqueous solution of gold(III) chloride hydrate (0.3 mM) was added five times at 2-minutes intervals. Finally, the sample was rinsed with water and dried under a stream of argon.

2.3. Immobilization of the Protein

Wild-type *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 (Stanford University, CA) [42]. RCs were purified according to a modification of the original method [29]. The bc_1 complex poly-his-tagged on the C-terminal end of the cyt *b* subunit was expressed and purified according to Crofts et al. [43]. The immobilization of the proteins on the two-layer gold surface on top of the ATR crystal was performed according to a method described earlier [9] and 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 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 µM DiPhyPC in DDM-DPK). In the case of additional ubiquinone, Q-10 was solubilized together with DiPhyPC (6 µM Q-10 in DiPhyPC/DDM-DPK). After incubation, DDM was removed by adding Bio-beads to the lipid-detergent solution. An idealized picture of the final structure is shown in Fig. 1

2.4. ATR-SEIRA Spectroscopy (ATR-SEIRAS)

SEIRA spectroscopy was performed in a flow cell, originally designed for electrochemical excitation of the protein, which was mounted on top of a trapezoid single reflection silicon ATR crystal. The IR beam of the FTIR

spectrometer (VERTEX 70v, from Bruker, Ettlingen, Germany) was coupled into the crystal at an angle of incidence $\Theta = 60°$ by using the custom-made setup described previously [17]. 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. To establish anaerobic conditions within the flow cell, the DPK solution was flushed with argon for 20 minutes, after which a chemical oxygen trap consisting of glucose (0.3% w/w), glucose oxidase (75 µg/mL) and catalase (12.5 µg/mL) was added.

Thereafter IR measurements were done under anaerobic conditions at 28°C. The sample chamber housing the flow cell was purged with dry, carbon dioxide-free air in order to remove CO_2 and water vapour from the light path. FTIR spectra were recorded at 4 cm^{-1} resolution using Blackham-Harris 3-term apodization and a zero filling factor of 2. The interferograms were measured in double-sided mode and transformed into spectra using the power phase correction mode. Spectra were analyzed using the software package OPUS 7 and Origin Lab's Origin software. Sample illumination was provided by white light from a Fiber-Lite DC950 illuminator (150 W, quartz halogen lamp) with an optical fiber from Dolan-Jenner (Boxborough, MA). The light intensity measured at 800 nm was 0.2 W/cm^{-2}.

3. Results and Discussion

3.1. SEIRA Spectra as a Function of Co-Immobilization Time

Co-immobilization of RC and the bc_1 complex via his-tags attached to the P side of the RC and the C-terminal end of the cyt *b* subunit, respectively, was monitored as a function of time (Fig. 2). For this purpose, we employed a two-layer gold surface, optimized with respect to the enhancement effect of nano-structured gold surfaces [17] (Fig. 1). Immobilization is indicated by an increase of vibrational components in the amide I region with a maximum at 1647 cm^{-1} as in the case of RC alone. The presence of the bc_1 complex is indicated by an additional band at 1592 cm^{-1}, which has been attributed to the ν_{37} vibration of hemes b_L and b_H [18] or, alternatively, to the ν(C=C) vibration of quinone [19]. Protein films of the bc_1 complex alone, dried and rehydrated on an ATR crystal, also showed characteristic bands at 1649 and 1540 cm^{-1} attributed to amide I and II vibrations [20]. Bands around 1567 cm^{-1} are generally assigned to N-H bending and C-N stretching vibrations of the amide II band. However, bands around 1590 cm^{-1} were also found in SEIRA spectra of other his-tagged proteins together with small bands at 1438 cm^{-1} with a shoulder at 1422 cm^{-1}, which have been attributed to the rearrangement of NTA moieties [21].

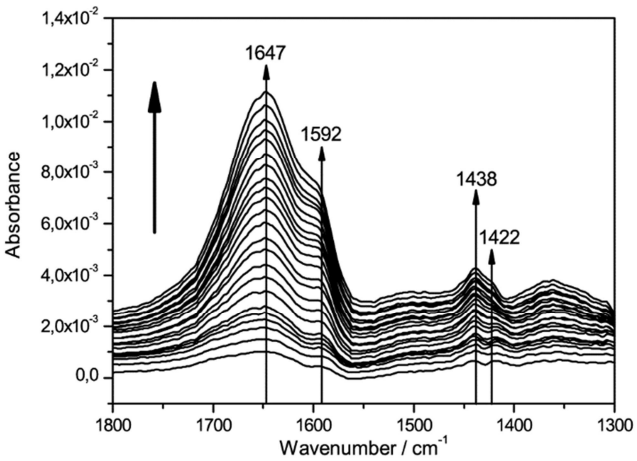

Figure 2. *SEIRA spectra of RCs co-immobilizing with bc_1 complexes to the gold surface as a function of time. Total adsorption time is 4 h. Time intervals between recordings are 10 min.*

3.2. Light-Minus-Dark SEIRA Spectra of the Co-reconstituted Proteins

Light-minus-dark difference spectra were recorded under continuous illumination of both proteins reconstituted in the ptBLM. A steady state was not obtained on the seconds time scale as described by Breton, probably because different from our experiment they used an electron donor to reduce the oxidized primary special pair, $P870^+$ or P^+ back to P [12]. Moreover, the overall appearance of the spectra is strikingly different from steady state FTIR difference spectra found in the literature, which usually are composed of narrow peaks and troughs [12]. Most of the unfamiliar features of our spectra can be explained in terms of the theory of SEIRAS, notably the strong dependence on the orientation of the transition dipole moments, which must be oriented perpendicular to the surface. This is expressed in the surface selection rule shown to be valid in the context of SERRS as well as of SEIRAS, predicting that bands are the stronger the more the transition dipole moment of the respective functional group is oriented perpendicular to the surface [8, 22]. This means that the same component present in different orientations may be strongly represented or not at all. This largely accounts for the different sensitivities of bands associated with the same functional group, e.g. bands that represent QH_2 at 1434, 1491, 1470 cm^{-1}, of which only 1434 cm^{-1} is definitively seen here. The same holds for the bands that represent P^+ modes such as 1750, 1704, 1550, 1480, 1283 and 1295 cm^{-1}, of which only 1283 cm^{-1} is seen. This effect is strongly enhanced by the pre-orientation of the RC molecules due to his-tag binding followed by reconstitution into a lipid bilayer. This arrangement has to be compared to solubilized RCs used previously for FTIR after drying and rehydration [12]. Nevertheless, the single bands can be tentatively assigned to certain modes on the basis of FTIR spectra known from the literature, see Table 1. Almost all the bands represent products of the illumination, which explains the fact that mostly positive bands are recorded. For a more detailed discussion of the bands see further below.

Absorbances of all the bands thus obtained under continuous illumination increased on a time scale of minutes similar to the observation made with the RC alone [9]. For an explanation of the long time scale, see further below. The spectrum taken after 5 min illumination time of co-reconstituted bc_1 complex and RC is shown in Fig. 3, and compared with the respective spectra of the RC alone obtained under the same conditions.

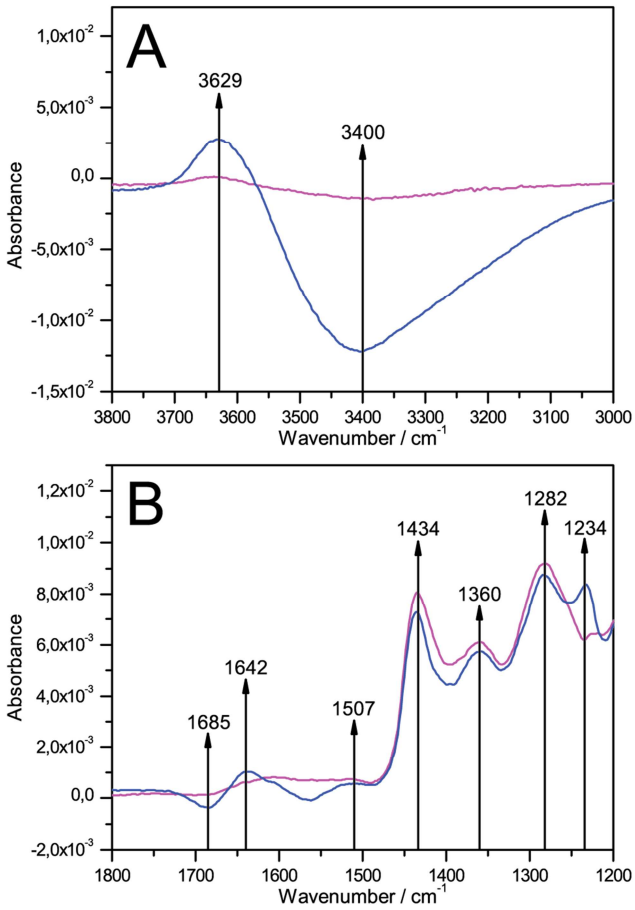

Figure 3. *Light-minus-dark spectra under continuous illumination, taken after 5 min illumination of RC alone (blue line) and RC co-reconstituted with bc_1 complex (magenta line) in the ptBLM, in the upper (A) and lower (B) wavenumber region.*

In the presence of the bc_1 complex, absorbances of the bands at 1282, 1360, 1434 cm^{-1} are almost unchanged whereas the bands at 1234, 1507, 1642 and the negative band at 1685 cm^{-1} are almost absent. Moreover, the broad negative and positive bands at 3400 and 3629 cm^{-1} exhibit a much smaller absorbance compared to the RC alone. The negative band at 1685 cm^{-1} may reflect the decrease of the ground state of the primary donor, P [13, 23], whereas the band at 1642 cm^{-1} could reflect the C=O vibration of ubiquinone, however, strongly overlapping with the water bending vibration [12, 13, 16, 24-26]. The band at 1234 cm^{-1}, a prominent band also in the absence of the bc_1 complex, could not be correlated to any kind of alteration. It lies in the region of C-O stretching vibrations of protonated carboxylic acids [27]. A band at 1238 cm^{-1} has been assigned to the δ (COH) vibration of tyrosine or, alternatively, to the ν_{as} (PO_2) vibration of certain lipids [18, 19]. The band at 1507 cm^{-1} has been reported by Leonard and Mäntele [23] in FTIR spectra of the RC, but was not assigned to any vibration. The bands at 1282 and 1434 cm^{-1} have been assigned to the P^+ species of the primary donor [12, 25], and QH^2 [28, 29], respectively, and were shown previously to be formed slowly over time under continuous light excitation of the irrespective of the presence of the bc_1 complex. The band at 1360 cm^{-1} has been assigned to the δ (CH_3) vibration of the methyl group at the 5-position of the ring of the semiquinone difference spectra, Q_A^--minus-Q_A and Q_B^--minus-Q_B, the electron acceptors of the RC. It was also found to increase during continuous illumination of RCs alone [30, 31]. Broad positive and negative bands in the region 3400-3650 cm^{-1} have been attributed by Iwata et al. [14] to water stretching vibrations associated with semiquinone formation at the Q_A and Q_B sites of the RC. The bands at 3400 and 3629 cm^{-1} observed here are most akin to those of Q_B/Q_B^-. Changes of these bands under continuous illumination are considerably smaller in the presence of the bc_1 complex. The same applies to the band pair 1642/1685 cm^{-1}. The band at 1642 cm^{-1} is in the amide I region but has also been assigned to ubiquinone and the re-orientation of water molecules, which would be consistent with the decrease of the bands at 3400 and 3629 cm^{-1}. The negative band at 1685 cm^{-1} could reflect the decrease of the ground state of the primary donor, P [13, 23], which would decrease on illumination, consistent with the appearance of the P^+ species indicated by the positive band at 1282 cm^{-1}. Tentative band assignments are collected in Table 1. The very slow evolution of spectra in the absence of an electron donor was explained earlier [9] in terms of an inter-protein reaction between two RC molecules

$$2\ P^+Q_B^- + 2H^+ \rightarrow P^+Q_B + P^+Q_BH_2 \qquad (1)$$

Following the light excitation of the RC consisting of electron transfer between the primary electron donor (special pair, P870 or P) and the electron acceptors Q_A and Q_B

$$PQ_B + hv \rightarrow P^+Q_B^- \qquad (2)$$

Eq (1) is followed by release of QH_2 into the membrane and rebinding of Q_B from the quinone pool. Mechanistically, eq (2) will involve transient states such as $P^+Q_A^-$ and $P^+Q_A^-Q_B^-$, with coupled protonation events [35]. Hence, instead of reaching a steady state on the level of $P^+Q_B^-$, species such as P^+ and QH_2 (and/or Q_BH_2, as indicated by bands at 1282 cm^{-1} for P^+ [12, 25] and 1434 cm^{-1} for QH_2 [28, 29]) could be formed on the time scale of minutes. In the presence of the bc_1 complex, this slow inter-protein reaction (eq 1) described in [9] seems to go on almost unimpeded, as illustrated by Fig. 3. Only the bands indicating the disappearance of P (1685 cm^{-1}) and UQ (1642 cm^{-1}), as well as the water stretching vibrations associated with semiquinone formation bands, are smaller, which can be explained in terms of eq. 1 followed by protonation of Q_B^{2-} and release as QH_2 into the membrane, with rebinding of Q_B from the quinone pool. This reaction is considered to depend

on the concentration of Q within the lipid phase. With no Q-10 added this concentration will be determined by the amount of UQ which stays with the membrane protein during purification. In the case of the RC, there is always some UQ left during purification as we have seen in [9]. The preparation of the bc_1 complex, on the other hand, apparently does not contribute to the concentration of UQ and hence the total concentration within the mixed layer is smaller. This can explain the disappearance of the band pair 1685 and 1642 cm^{-1} within the mixed protein layer as compared to the homogeneous layer of RCs. In order to support this explanation, we have co-reconstituted additional lipophilic Q-10 together with the two proteins. The absorbance of all the bands was increased (Fig. 4) and more importantly, absorbances of the band pair 1685/1642 cm^{-1} were restored when the spectrum was taken under otherwise the same conditions (Fig. 4).

Table 1. *Tentative band assignment of bands of RCs co-reconstituted with bc_1 complexes in the ptBLM under continuous illumination.*

Band position [cm^{-1}]		**Tentative Assignment**	
Experimental	**Literature**	**Moiety**	**Vibrational Mode**
1282	1282 [23, 25, 32]	P^+	(complex)
1360	1355 [3], 1365 [31]	Q_A	δCH_3
1434	1433 [13, 28, 33, 34]	QH_2	
1642	1640 [25, 26], 1641 [12, 16, 24], 1642 [13]	Quinone – Q, Q_B	C=O
1685	1682 [23], 1683 [13]	9-keto group of P	C=O
3400	3485 [14]	Q_B^-/Q_B or P^+	H_2O
3629	3632 [14]	Q_B^-/Q_B or P^+	H_2O

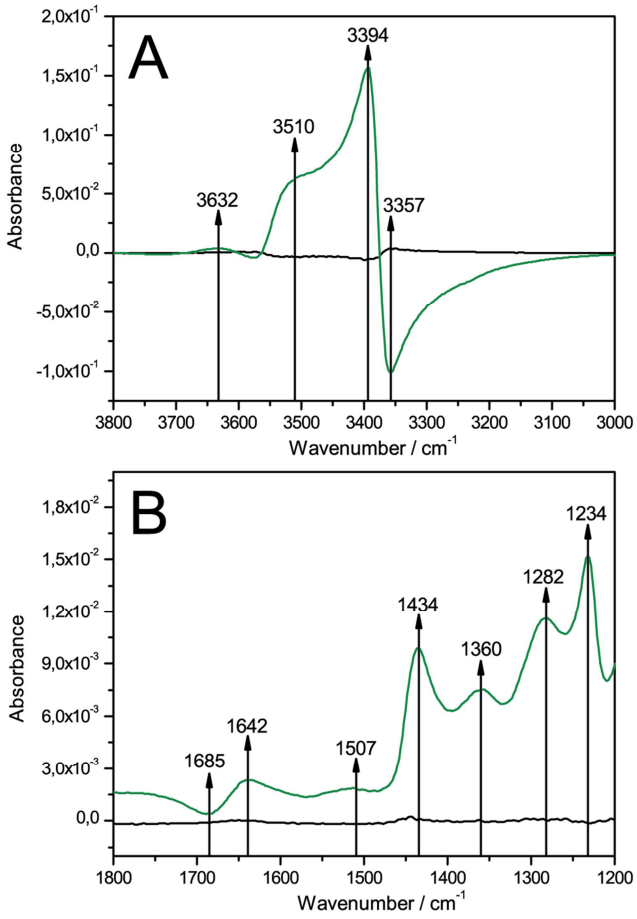

Figure 4. *Light-minus-dark spectra under continuous illumination taken after 5 min illumination of RC co-reconstituted with bc_1 complex and additional Q-10 (green line) in the ptBLM, in the upper (A) and lower (B) wavenumber region. The black line is the dark-minus-dark difference spectrum.*

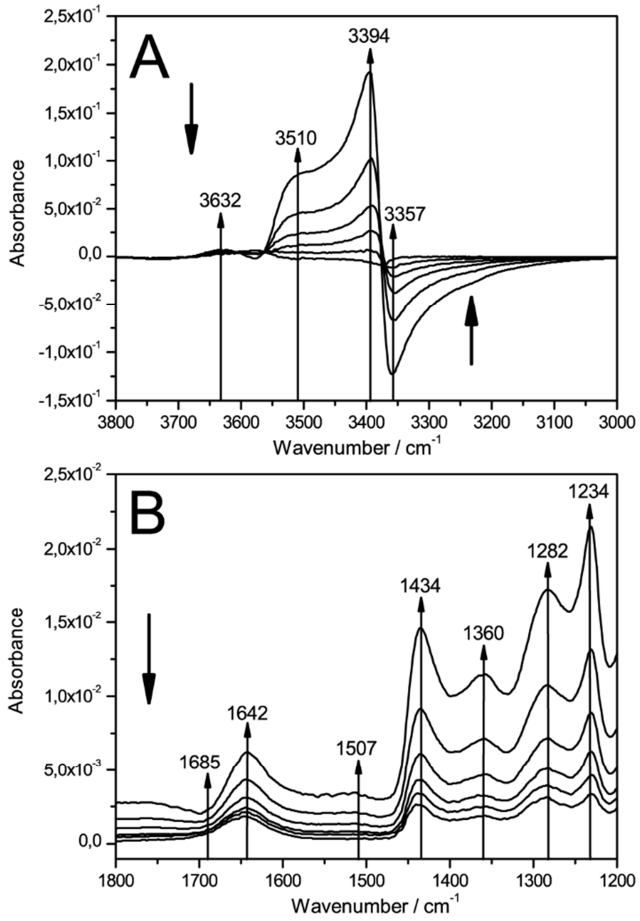

Figure 5. *Decrease of characteristic bands during relaxation in the dark. Spectra were recorded every 10 min.*

Moreover, the bands in the region 3600 – 3300 cm^{-1}, interpreted by Iwata et al. [14] in terms of water stretching vibrations associated with semiquinone formation, undergo a dramatic change in appearance. The small peak at 3632 cm^{-1}

may be a remnant of the 3629 cm^{-1} band seen in the absence of bc_1 complex. Additional bands are now observed at 2851, 2922, 2958 cm^{-1}, which are not seen before co-reconstitution of additional Q-10. They could be due to CH stretching vibrations of the hydrocarbon residue of Q-10. Alternatively, it may reflect changes in the water spectrum in response to changes in membrane structure or surface architecture due to the presence of Q-10. The band at 1234 cm^{-1}, which is negligible in the absence of added Q-10, is substantially enhanced with Q-10 added. The state obtained after five minutes illumination time was then permitted to relax in the dark (Fig. 5).

All the bands decreased over time including the 1642/1685 cm^{-1} band pair, as illustrated in the plots of the band areas vs. time of relaxation (Fig. 6).

The almost complete disappearance, after switching off the light, of all the bands formed during continuous illumination supports the explanation given above for the slow inter-protein reaction between RC molecules. Relaxation can be explained in terms of the reversal of the original disproportion, eq (1)

$$2P^{+}Q_B + QH_2 \rightarrow P^{+}Q_B^{2-} + P^{+}Q_B \rightarrow 2\ P^{+}Q_B^{-}, \qquad (3)$$

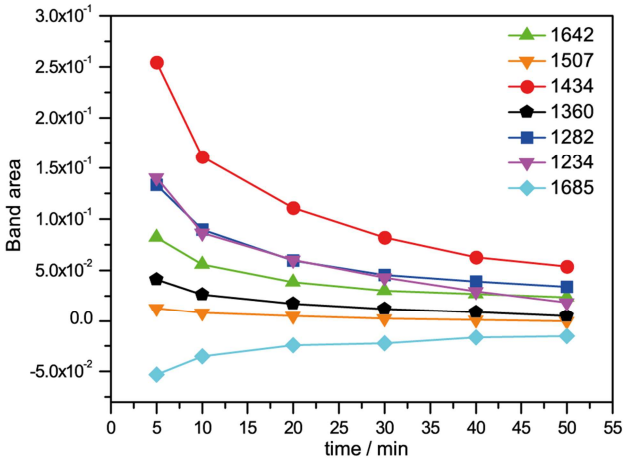

Figure 6. *Kinetics of characteristic bands during dark relaxation. Red circles 1434 cm^{-1}, blue squares 1282 cm^{-1}, green up triangles 1642 cm^{-1}, violet down triangles 1234 cm^{-1}, black diamonds 1360 cm^{-1}, orange down triangles 1507 cm^{-1}, light blue squares 1685 cm^{-1}. All bands are approaching zero.*

where the rates of both forward and backward reactions are limited by the exchange of Q and QH_2 species between protein and lipid phase. Local concentrations of quinone species rather than diffusion coefficients are considered to control reaction rates, as shown in other instances [36].

Finally, light-minus-dark absorbance spectra were recorded not only in the presence of additional Q-10 but also with (oxidized) cyt *c* added to the aqueous phase (Fig. 7).

As a result, we observe a drastic change in the behavior of the system. Just as in the experiment described by Breton [12], it quickly arrives at a stationary state characterized by bands that are much smaller than those obtained in the experiments without cyt *c* (Fig. 7). However, the bands below 1460 cm^{-1} are essentially identical in position and relative amplitude to those seen in the absence of cyt *c* (compare Fig. 4).

The stationary state indicates a fast interaction of the light-activated RC with the bc_1 complex, but only in the presence of cyt *c* in the aqueous phase. This suggests that electrons delivered to the bc_1 complex by QH_2 are used to reduce cyt *c*. In addition, the bands at 3632, 3510, 3394, 3357 cm^{-1} disappeared entirely, whereas a band at 3454 cm^{-1} appears, which resembles a shifted version of the band at 3400 cm^{-1} seen in the absence of bc_1 (Fig. 3A). These effects indicate an appreciable re-arrangement of water molecules associated with semiquinone formation at the Q_A and Q_B site of the RCs [14]. Moreover, the band around 1642 cm^{-1} has disappeared and two new bands, at 1660 and 1544 cm^{-1}, are seen, characteristic for amide I and amide II vibrations, respectively, usually indicating changes in the protein secondary structures.

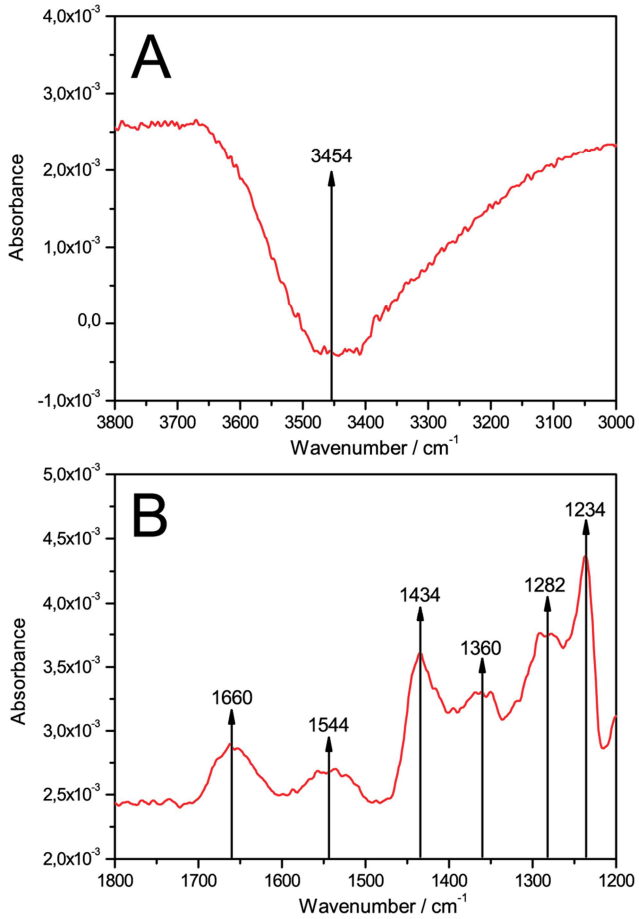

Figure 7. *Light-minus-dark spectra of RC co-reconstituted with bc_1 complex and additional Q-10 in the ptBLM under continuous illumination after addition of (oxidized) cyt c, in the upper (A) and lower (B) wavenumber region. Compare Fig. 4 for the same experiment without cyt c. Note the different scales of the y axis.*

4. Conclusions

Due to the position of the his-tags, the RC and the bc_1 complex are oriented with the primary donor and the C-terminal end of the cyt *b* subunit directed toward the gold film, respectively (Fig. 1). In this configuration, the cyt *c*

binding side of the two proteins is located on opposite sides of the membrane. This is different from photosynthetic bacteria, where cyt *c* is reduced and re-oxidized within the lumen of chromatophores, by c_1 via the Rieske iron sulfur center (ISP) of the bc_1 complex and by P^+ of the RC, respectively, while cyt *c* shuttles between the two complexes. This configuration, however, could not be realized at the time of this study.

Nevertheless, in the present configuration the bc_1 complex presents the cyt *c* binding site to the outside of the membrane, thus allowing cyt *c* added to the surrounding solution to interact directly with the bc_1 complex but not with the RC. The decrease of the bands at 1282 and 1434 cm^{-1} (attributed to P^+ and QH2, respectively) is explained in terms of the inter-protein reaction eq (1) that is inhibited in the presence of cyt *c*. We conclude that the bc_1 complex turns over, however, to an extent that is controlled by the amount of QH_2 thus transferring electrons to cyt *c*. We consider that during this turnover a species may be formed that is able to reduce P^+ back to P, i.e., an electron donor just as in the experiment described by Breton [12]. We have to conclude that such a species is absent before the addition of cyt *c* because otherwise the disproportionation reaction eq (1) would proceed undisturbed. Nevertheless, some QH_2 will be available together with fully oxidized cyt *c* present in the aqueous phase adjacent to cyt c_1 of the bc_1 complex, while P^+ is still oxidized. These are the conditions under which the bc_1 complex will undergo the well known Q-cycle mechanism [37].

The first and rate limiting step in the Q-cycle is the oxidation of QH_2 in the Q_o site by the ISP. The SQ formed at center Q_o is highly unstable [38] 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 QH_2 [37, 39]. Hence, after the first turnovers we expect three possible reductive species as candidates for P^+ reduction: SQ at center Q_o, SQ at center Q_i and reduced cyt *c*. We consider the most likely candidate to be the SQ at center Q_i, as the Q_o site SQ is well protected and very short lived [38]. Moreover, in the present configuration, center Q_i is located closer to P/P^+ than center Q_o (Fig. 1). Cyt *c* is a highly unlikely candidate because the illumination of the RC yields only P^+ and semiquinone Q_B^- and the second electron can be transferred to Q_B^- only when P^+ is re-reduced to P [15]. This would result in a continuous formation of QH_2 as well as a continuous turnover of the both the RC and the bc_1, which obviously does not happen.

The assumption that the reducing species for P^+ is semiquinone is supported by the finding that the relative amplitudes of the 1282 and 1434 cm^{-1} bands are almost unchanged, albeit much smaller in the presence of cyt *c*. This can be explained in terms of the reduction of P^+ at the level of $P^+Q_B^-$ which controls the disproportionation reaction eq (1) and hence the formation of P^+ and QH_2 in equal amounts. The reduced amount of QH_2 in turn controls the reaction of the bc_1 with cytochrome c until both reactions balance eachother in a stationary state. We conclude that the SQ formed at the Qi site of the bc_1 reduces $P^+Q_B^-$ thereby controlling both the disproportionation reaction eq (1) and the turnover of the bc_1 in agreement with our observation. This conclusion implicates a certain mobility of the SQ considered previously [40]. We conclude that the stationary state attained in the presence of cyt *c* is a strong indication of the interaction of the bc_1 with QH_2.

The stationary state finally obtained in the presence of cyt *c* can be compared with those obtained in previous FTIR studies of the RC reported by Breton in the presence of an electron donor [12]. They reported bands at 1657 and 1549/1533 cm^{-1}, which were attributed to conformational changes of the protein backbone of RCs as a consequence of semiquinone formation. A broad band in the spectral range 3200-2200 cm^{-1} was also reported by the same author, corresponding to broad continuum bands previously assigned to excess protons in hydrogen bond networks. Strong bands at 1653 and 1544 cm^{-1} were also shown in perfusion-induced ATR-FTIR redox difference spectra particularly of the ISP of the bc_1 complex from *R. capsulatus* [41]. Therefore, the appearance of the bands at 1660 and 1544 cm^{-1} support the notion of a turnover of the bc_1 complex as a consequence of RC excitation.

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Abbreviations

RC: Photosynthetic Reaction Center, SEIRAS: Surface-Enhanced Infrared-Absorption Spectroscopy, ptBLM: protein-tethered bilayer lipid membrane, cyt *c:* cytochrome *c*, Q: ubiquinone, QH_2: ubiquinol, SQ: semiquinone

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