Surface-Enhanced Infrared Absorption Spectroscopy (SEIRAS) of Light-Activated Photosynthetic Reaction Centers from *Rhodobacter sphaeroides* Reconstituted in a Biomimetic Membrane System

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ABSTRACT: Surface-enhanced IR absorption spectroscopy (SEIRAS) in the ATR configuration has been performed on reaction centers (RCs) from *R. sphaeroides*. Surface-enhancement is achieved by a thin, structured gold film present on the surface of an ATR crystal. Purified RCs are immobilized as a monolayer on top of the gold film via a poly his-tag engineered to the C-terminal end of the M subunit. Subsequently, the RCs are reconstituted into a lipid bilayer by *in situ* dialysis. Light-minus-dark absorbance spectra were recorded under continuous illumination using the spectrum in the dark as the reference. A number of strong bands have been observed indicating the excitation of the special pair as well as alterations of quinone/quinol species. Spectra were recorded at different time intervals with and without liposoluble Q_{10} co-reconstituted into the lipid phase. A steady (photostationary) state was approached slowly and bands were found to increase or decrease reversibly on illumination and relaxation. Tentative assignments were made for some bands, based on previous FTIR measurements. The long time scale of these processes was tentatively explained in terms of inter-protein reactions of RC molecules.

Keywords: Photosynthetic reaction center, *Rhodobacter sphaeroides*, purple bacteria, SEIRAS

INTRODUCTION

FTIR spectroscopy is well suited to investigate photoexcitation of bacterial reaction centers (RCs). RCs have been investigated either in chromatophores[1] of the respective bacteria or in purified and solubilized form, mostly as a rehydrated thin film between two CaF_2 windows.[2-8] Marker bands for quinol formation were identified, obtained from the quinone/quinol pool present within chromatophores or added to solubilized RCs. Steady-state, light-minus-dark difference absorbance spectra under continuous illumination[5,9-12] and time-resolved spectra[1,3-4,7,13] have been measured. In the majority of these studies, the RCs have been observed in the presence of an electron donor to reduce the oxidized primary electron donor, $P870^+$. [1,7-9,12,14] Other redox active compounds have also been added as redox mediators.[1,7,9,12,14]

We use a different strategy using surface-enhanced IR absorption spectroscopy (SEIRAS) according to which FTIR spectra are measured in the ATR configuration, with a thin structured gold film on the ATR crystal to achieve enhancement of the IR signal. Purified RCs can be immobilized as a monolayer on the surface of the gold film via his-tag, a technology also used for the measurement of photocurrents.[15-18] In previous SEIRAS studies of cytochrome *c* oxidase (C*c*O), we had extended this strategy by *in situ* dialysis of the protein monolayer in the presence of lipid micelles. The protein is thus reconstituted in a lipid bilayer to form a protein-tethered bilayer lipid membrane (ptBLM) (Fig. 1).[19-20] As a benefit of this structure, liposoluble Q_{10} can be co-reconstituted into the lipid phase, while the protein is preserved in a fully functional form.

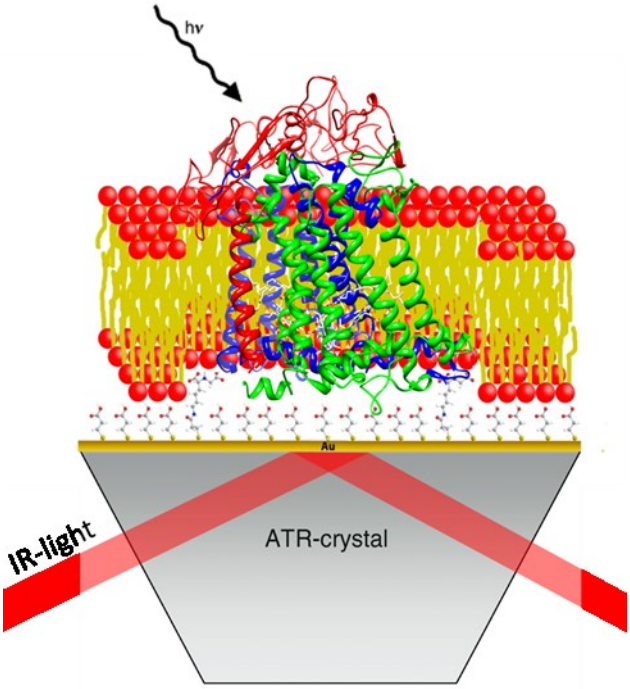

Figure 1. Setup of RCs immobilized on NTA-functionalized gold surface and reconstituted in the ptBLM. RCs are immobilized with the special pair oriented towards the surface of a silicon ATR crystal, covered with a two-layer gold film.

MATERIALS AND METHODS

Solvents and Chemicals. Deionized water was used from a Sartorius-Stedim system (Goettingen, Germany) with a resistivity of 18 MΩ cm. Argon 4.8 was obtained from Linde Gas GmbH (Stadl-Paura, Austria). 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 Coenzyme Q_{10} (Q-2, diisoprenyl-ubiquinone) were purchased from Sigma-Aldrich (Steinheim, Germany). All chemicals were used as purchased.

Preparation of the two-layer gold surface on the ATR crystal. Preparation was done as previously described by Nowak et al.[21] 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 crystal was dried under a stream of argon and annealed at 100 °C for 60 minutes. The crystal was allowed to cool to room temperature, then 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. After that the Au surface clearly showed protruding structures ranging in diameter from 43-60 nm (average 57 nm) with an aspect ratio of around 12. Finally, the crystal was rinsed with water and dried under a stream of argon.

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.[22] RCs were purified according to a modification of the original method.[23] The immobilization of RC on either the template stripped gold (TSG) surface (see supplementary information) or the ATR crystal was performed according to a method described by Nowak et al.[24] and references therein. TSG was used for Surface Plasmon Resonance (SPR)

Spectroscopy and Electrochemical Impedance Spectroscopy (EIS) (see supplementary information). 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-DPK buffer (0.05 M K_2HPO_4, 0.1 M KCl, pH 8, 0.1% DDM) before starting the SEIRA measurements. SEIRA spectra (Fig. 2) were taken while RCs dissolved in DDM-DPK were adsorbed to the NTA-functionalized gold surface at a final concentration of 100 nM. After 4 h time of adsorption carried out at 28°C, the cell was rinsed with DDM-DPK to remove unspecifically 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). In both cases, DDM was removed by adding Bio-beads to the lipid-detergent solution.

ATR-SEIRA-Spectroscopy. The electrochemical cell 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.[21] 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. IR measurements were done under aerobic conditions at 28 °C. The sample unit was purged with dry, carbon dioxide-free air. 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 OriginLab's Origin software.

Illumination was performed with white light from a Fiber-Lite DC950 illuminator (150 W, quartz halogen lamp) obtained from Dolan-Jenner (Boxborough, MA) with a light intensity of 0.2 W/cm^{-1} (at a wavelength of 800 nm).

RESULTS AND DISCUSSION

Formation of the ptBLM. Immobilization of the RCs and subsequent formation of the ptBLM followed by Surface Plasmon Resonance (SPR) and Electrochemical Impedance Spectroscopy (EIS) is shown in Figs. S1, S2A and B, respectively. Optical thickness and electrical parameters given in Table S1 correspond to the respective data found in the case of C*c*O, where the surface concentration was determined to be ~6 pMol cm^{-2}, in agreement with the calculated value for a densely packed monolayer from the crystal structure of C*c*O from *R. sphaeroides.* [19,24-25] We conclude that a monolayer of RCs of a similar packing density had been formed on the gold film, with only small voids left between single proteins to be filled with a lipid bilayer.

SEIRAS measurements as a function of illumination. SEIRA spectra were recorded as a function of time in the course of the binding of the RCs via his-tag attached to the P side of the protein (Fig. 2).

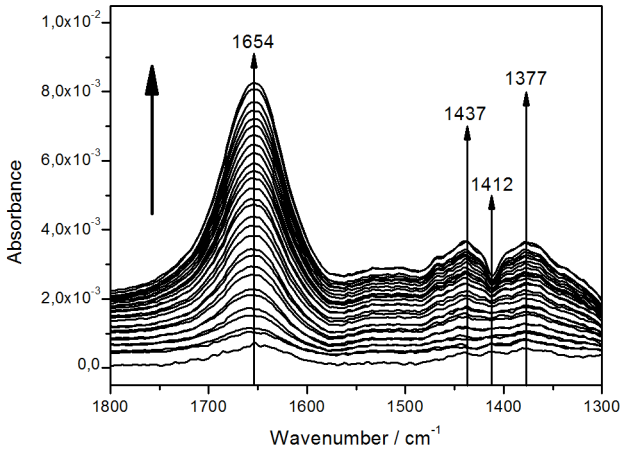

Figure 2. SEIRA spectra of RCs in the course of their immobilization on the gold film as a function of adsorption time. Total adsorption time was 4 h, intervals between spectra were 10 min. The spectrum of the functionalized gold surface was used as the reference.

Vibrational components of α-helices in the amide I region can be recognized at 1654 cm^{-1}, as well as two bands at 1437 and 1377 cm^{-1}. The negative band at 1412 cm^{-1} may be due to changes in the NTA structure/orientation during complex formation with the his-tag.[26] No amide II bands are seen in the spectra. This can be explained in terms of the theory of SEIRAS, predicting that dipoles oriented perpendicular to the surface are subject to a particularly high surface-enhancement effect, while dipoles pointing in other directions are not detected.[27] Moreover, the enhancement decreases with distance from the metal film. The RCs are arranged on the surface with the α-helices pointing in the z-direction, giving rise to the relatively high absorbance of the amide I band. The transition dipole moment for the amide II mode is roughly orthogonal to this, with negligible SEIRA enhancement and consequently much weaker absorbance.

Next, light-minus-dark difference spectra were recorded under continuous illumination of the RC reconstituted in the ptBLM, first without any additional Q_{10}. A total of 1000 scans were recorded and averaged for every spectrum. Surprisingly, a steady state was not obtained on the seconds time scale, as described by Breton,[6] for example. Instead, absorbances of many bands increased

over a much longer time scale. The first spectrum taken after 5 min illumination time, with no additional Q_{10}, is shown in Fig. 3. Next, spectra were recorded at 5 min intervals, also without additional Q_{10} (Fig. 4), and band areas of selective peaks were plotted as a function of time (Fig. 5A). A steady state is approached after > 20 min.

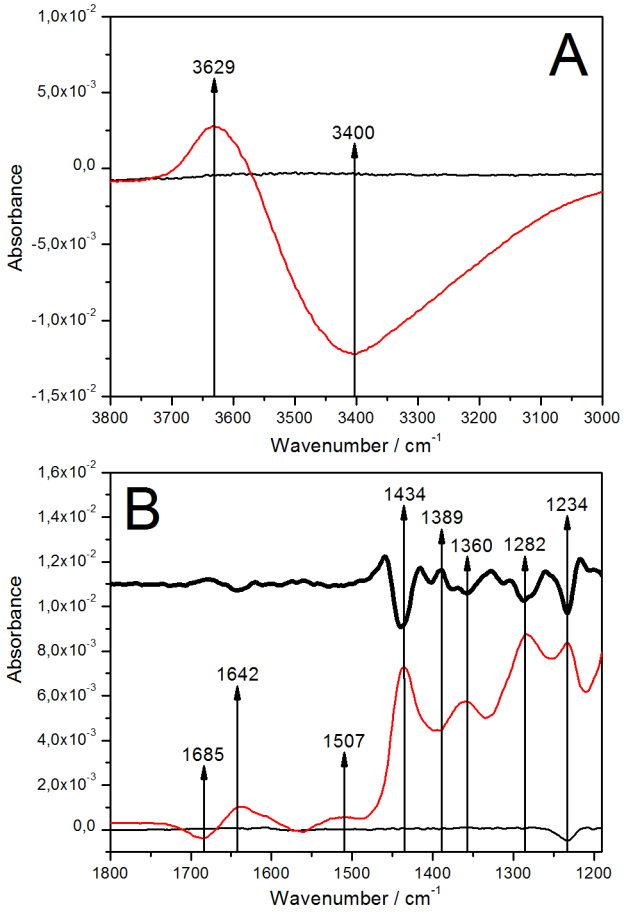

Figure 3. Light-minus-dark absorption spectra of RCs immobilized in the ptBLM after 5 min illumination time (red line), with the spectrum in the upper (A) and lower (B) wavenumber region. The reference spectrum in each is dark-minus-dark, which did not change as a function of time without illumination. The bold line in (B) represents the second-derivative spectrum showing a negative band appearing at 1389 cm^{-1} between the 1434 and 1360 cm^{-1} bands. The second derivative also shows that the band at 1642 cm^{-1} is an overlap of a variety of bands, which, however, cannot be resolved into single bands.

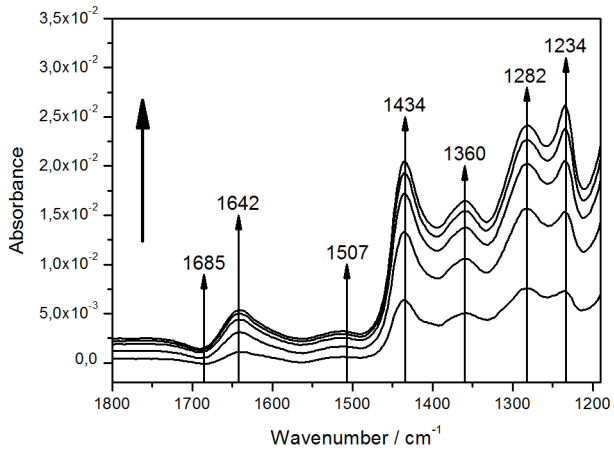

Figure 4. Time evolution of light-minus-dark absorption spectra of RCs immobilized in the ptBLM without added Q_{10} during the course of continuous illumination. Difference spectra were recorded every 5 min.

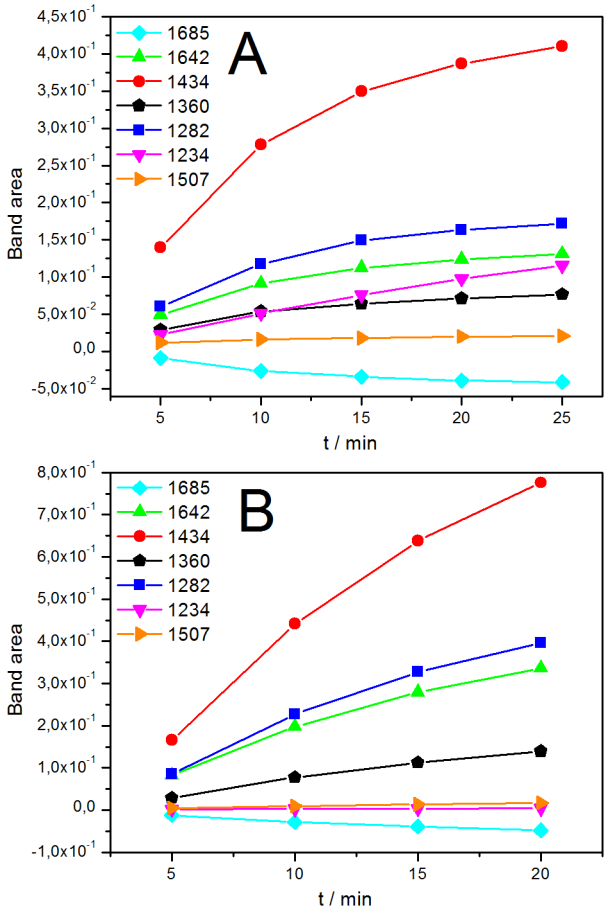

Figure 5. Band areas of absorbances of characteristic bands under continuous illumination of RCs in the ptBLM taken at different time intervals without (A) and with added Q_{10} (B).

As seen in the spectra, a number of positive bands appear at 1234, 1282, 1360, 1434, 1507, 1642 and a negative band at 1685 cm^{-1}. Between the bands at 1360 and 1434 cm^{-1} a small negative band is buried at 1389 cm^{-1} but is seen in the second derivative spectrum also shown in Fig. 3B. Broad negative and positive bands appear at 3400 and 3629 cm^{-1}, respectively. The most prominent band at 1434 cm^{-1} is strongly indicative of a band assigned to QH_2 by FTIR,[1-2,4,7] although associated bands at 1491, 1470 and 1375 cm^{-1} are not seen. An FTIR band at 1389 cm^{-1} is also attributed to QH_2, [1,7] but the negative sign here is not consistent with the generation of QH_2 indicated by the positive band at 1434 cm^{-1}. The band at 1282 cm^{-1} is an FTIR marker band of P^+.[28-30] A band at approx. 1360 cm^{-1} has been assigned to the δCH_3 vibration of the methyl group at the 5-position of the ring in the semiquinone difference spectra, between Q_A^- and Q_A and Q_B^- and Q_B.[11,31] FTIR bands indicating semiquinone were reported to occur at 1446 and 1479 cm^{-1}, which we do not see in our spectra.[11,31] The band at 1642 cm^{-1} may be an overlap of several bands in the region of the amide I band of α-helices and H-O-H bending vibration of water, and the C=O stretching vibration of Q_B is also assigned in this region.[4-6,29,32-33] Negative evolution of the band at 1685 cm^{-1} attributed to the C=O stretching vibration of the 9-keto group of P[4,30] is consistent with a decrease in the concentration of P.

Broad positive and negative bands in the region 3800-3000 cm^{-1} in FTIR were interpreted by Iwata et al.[14] as water stretching vibrations associated with formation of either semiquinone, Q_A^- or Q_B^-. Given the orientation of the RC in the ptBLM, with P facing the gold surface, oriented water(s) close to P/P^+ are also a likely a source of these bands. It is noteworthy that the spectra exhibit a well-defined isosbestic point.

An assignment for the band at 1507 cm^{-1} could not be found in the literature. A band at 1234 cm^{-1} also could not be found in the literature dealing with RCs, but lies in the region of C-O stretching

modes of carboxylic acids.[34] The latter, however, is a prominent recurrent band, which also remains after relaxation of all others (see below). It could not be correlated to any kind of alteration, for example addition of Q_{10}, time of illumination or relaxation, etc.

Tentative band assignments from previous FTIR measurements are collected in Table 1.

Table 1. Possible assignments of SEIRAS bands for RCs reconstituted in the ptBLM under continuous illumination

band [cm^{-1}]			tentative assignment	
experimental	FWHM	literature	species	component
1282	77.5	1282[28-30]	P^+	(complex)
1360	34.7	1355[11], 1365[32]	Q_A	δCH_3
1434	25.7	1433[1-2,4,7]	QH_2	
1643	66.7	1640[5,29],1641[6,32-33],1642[4]	quinone Q, Q_B	C=O
1685	35.2	1682[30],1683[4]	9-keto group of P	C=O
3400	312.7	3485[14]	Q_B^-/Q_B or P^+	H_2O
3629	194.5	3632[14]	Q_B^-/Q_B or P^+	H_2O

The origin of the bands discussed above was supported by spectra recorded with liposoluble Q_{10} co-reconstituted into the lipid phase. The absorbance of the strong bands was shown to increase under illumination and to decrease during dark relaxation (Fig. 6, 7, 8). Absorbances also increased when Q_{10} was co-reconstituted (Fig. 5B, 6), showing that the evolution of these bands is affected by the quinone/quinol pool. Summarizing, in the approach to steady state under continuous illumination, we detected the evolution of QH_2 and P^+ occurring in the time scale of minutes, which is fully reversible after switching of the light. The long time scale of the reactions will be discussed further below.

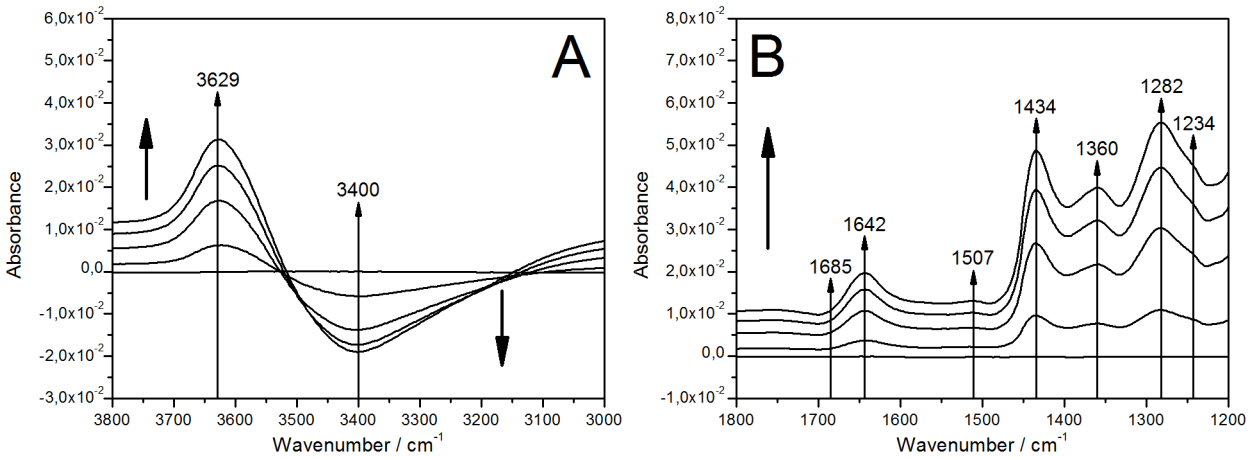

Figure 6. Light-minus-dark spectra of RCs in the ptBLM under continuous illumination with co-reconstituted Q_{10}, with the spectrum in the dark as the reference, in the upper (A) and lower (B) wavenumber region. Difference spectra were recorded every 5 min.

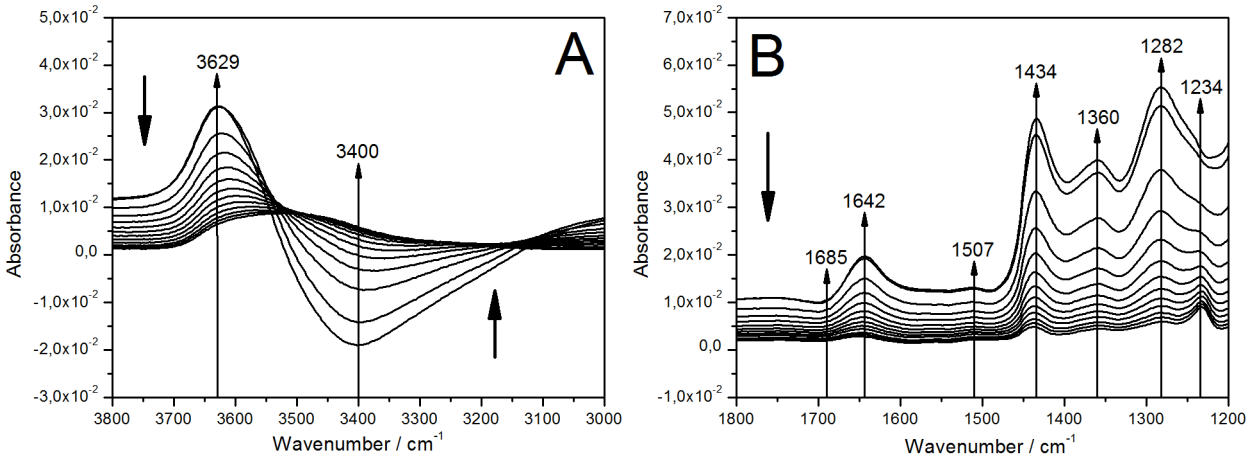

Figure 7. Relaxation of the bands of RC in the ptBLM with co-reconstituted Q_{10} after termination of continuous illumination in the upper (A) and lower (B) wavenumber region. Total relaxation time is 1 h, difference spectra were recorded every 5 min.

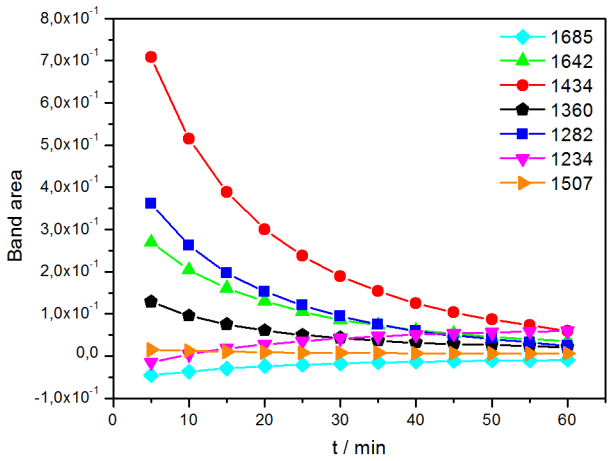

Figure 8. Kinetics of characteristic bands during relaxation. All band areas decrease during relaxation except for bands at 1234 and 1685 cm^{-1}, which increase.

CONCLUSIONS

Many of the bands found in our spectra can be correlated with bands reported in previous FTIR work (see Table 1). However, the overall appearance of the spectra is strikingly different from FTIR difference spectra found in the literature, which usually are composed of narrow peaks and troughs changing in the µs to s time scale.[29] Some of the unfamiliar features of our spectra can be explained by the theory of SEIRAS, notably the high sensitivity of vibrational components in close proximity to the surface, and the strong dependence on the orientation of the transition dipole moments, which must be oriented perpendicular to the surface.[27,35] 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. This effect is enhanced by the pre-orientation of the RC molecules within the ptBLM. Furthermore, the extreme dominance of positive bands throughout the spectrum may indicate a strong effect of the large, internal dipole moment of the RC in the charge-separated state. The

theory of SEIRAS, however, is not able to explain other features of our spectra, such as the substantial enhancement in band width, although this is also observed in other membrane proteins and we have speculated on possible origins, previously.[36]

The long time scale of the evolution of the SEIRA spectra, which we take to represent species such as P^+ and QH_2, is unexpected. In FTIR, the light-driven electron transfers to form Q_A^- and Q_B^- (and even Q_B^{2-} or Q_BH_2) have been studied extensively in the μs to s time range. However, the release of QH_2 from its binding pocket and its replacement by a quinone molecule from the quinone pool have received attention only relatively recently.[37-39] Multiple saturating flashes or continuous illumination of chromatophores from *R. sphaeroides* have revealed the slow accumulation of QH_2, but in the presence of ascorbate and other redox mediators.[1] This investigation was later extended to look at intermediate RC states, using rapid scan FTIR spectroscopy of detergent micelles of RCs.[7] Kinetic traces were analyzed in terms of a model taking into account the slow quinone exchange between RC micelles and pure detergent micelles, taking place in the time scale of seconds.[7,40-41]

In all of these studies, however, multiple excitations were possible because of the presence of electron donors to restore P^+ back to P. These are absent in our experiments. The slow time scale of the formation of species such as QH_2 (and/or Q_BH_2) and P^+ (as indicated by probable assignments from FTIR marker bands), in the absence of an electron donor, suggests the following possible explanation. Due to the high density of RCs, inter-protein quinone/semiquinone exchange may become possible, allowing release of quinol into the membrane. Under normal circumstances the states $P^+Q_A^-$ and $P^+Q_B^-$ recombine in 0.1-1 s and these would represent the only detectable states, depending on light intensity. At the high protein densities used here, however, it may be possible for some cross reactivity to occur, i.e. $P^+Q_B^-$ +

$P^+Q_B^- \rightarrow P^+Q_B + P^+Q_B^{2-}$, followed by protonation of Q_B^{2-} and release as QH_2 into the membrane, with rebinding of Q_B from the quinone pool. The resulting states, P^+Q_B and QH_2 can be expected to be quite long-lived and to accumulate slowly over time. The fact that all or most of the light-induced SEIRA bands exhibit similar kinetics is supportive of this model. During relaxation, restoration of the dark-adapted state would mostly likely be associated with direct reduction of P^+ by QH_2, yielding a short lived semiquinone that would reduce another P^+. However, reversal of the original disproportion is also possible: $2P^+Q_B + QH_2 \rightarrow P^+Q_B^{2-} + P^+Q_B \rightarrow P^+Q_B^- + P^+Q_B^-$.

Another characteristic of the SEIRA spectra is the full width at half maximum (FWHM) of the bands, which is significantly larger than that of the sharp bands found in the FTIR literature.[29] The FWHM is generally related to the freedom of movement of the structure associated with the particular component.[42-44] In previous studies, the protein was investigated either in the presence of an electron donor, or even an entire cocktail of mediators. In a previous SEIRAS study of cytochrome *c* oxidase we observed the narrowing of FWHM in the presence of mediators.[24,36] The effect might be explained in terms of the internal dipole potential of the naturally activated charge-separated state being markedly different from that after equilibration between mediators and redox sites.

The positive and negative bands in the region 3800-3000 cm^{-1} should be due to highly ordered water molecules[14] which are known to have particularly strong transition dipole moments. Remarkable in this context is a clear isosbestic point for these bands indicating that only *two* species vary in concentration to contribute to the absorption. The near-constancy of the isosbestic point also indicates that temperature effects should be small in this region.

In summary, SEIRAS of RCs present within a ptBLM yields a number of strong bands at wavelengths that correlate them with FTIR marker bands of photoexcitation. Weaker signals may

be buried underneath the strong bands, potentially contributing to the large, apparent FWHM of the major bands. The time-evolution of the SEIRA spectra is, however, unusually slow and not as observed in other experimental setups. The long time scale is consistent with the idea of an inter-protein disproportionation reaction followed by QH_2/Q exchange, which is diffusional. Reversal of the disproportionation accounts for the almost complete reversibility of the reactions observed under the experimental conditions used here.

ASSOCIATED CONTENT

Supporting Information. Additional Information about preparation of Template Striped Gold (TSG), Surface and Surface Plasmon Resonance Spectroscopy (SPR) measurements and Electrochemical Impedance Spectroscopy (EIS) used to monitor the immobilization of the protein and membrane formation. This material is available free of charge via the Internet at http://pubs.acs.org.

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