Photoreduction of the quinone pool in the bacterial photosynthetic membrane: identification of infrared marker bands for quinol formation

Alberto Mezzetti[a,b,1], Winfried Leibl[a], Jacques Breton[a], Eliane Nabedryk[a,*]

[a] *Service de Bioénergétique, Bâtiment 532, CEA Saclay, 91191 Gif-sur-Yvette Cedex, France*
[b] *Department of Physical Chemistry 'A. Miolati', University of Padova, Via Loredan 2, 35100 Padova, Italy*

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Abstract The photoreduction of the quinone (Q) pool in the photosynthetic membrane of the purple bacterium *Rhodobacter sphaeroides* was investigated by steady-state and time-resolved Fourier transform infrared difference spectroscopy. The results are consistent with the existence of a homogeneous Q pool inside the chromatophore membrane, with a size of around 20 Q molecules per reaction center. IR marker bands for the quinone/quinol (Q/QH_2) redox couple were recognized. QH_2 bands are identified at 1491, 1470, 1433 and 1388–1375 cm^{-1}. The 1491 cm^{-1} band, which is sensitive to $^1H/^2H$ exchange, is assigned to a C–C ring mode coupled to a C–OH mode. A feature at ~1743/1720 cm^{-1} is tentatively related to a perturbation of the carbonyl modes of phospholipid head groups induced by QH_2 formation. Complex conformational changes of the protein in the amide I and II spectral ranges are also apparent during reduction and reoxidation of the Q pool.

Key words: FTIR; Photosynthetic membrane; Quinone pool; Quinol

1. Introduction

The quinone/quinol (Q/QH_2) couple is a key player in bioenergetic processes such as respiration and photosynthesis [1]. These molecules function as electron carriers between membrane proteins, thereby coupling electron transfer to the formation of a transmembrane proton gradient. In photosynthesis, the reduction of Q to QH_2 takes place in special integral membrane proteins called reaction centers (RCs) and is induced by light. This allows triggering of the reaction by actinic light and makes photosynthetic membranes ideal model systems to investigate general principles governing the bioenergetic and structural roles of Q and QH_2 in proteins and membranes.

In RCs from purple photosynthetic bacteria, sequential absorption of two photons by a special pigment, the primary electron donor P, leads to the double reduction and protonation of a weakly bound ubiquinone, named Q_B, to ubiquinol Q_BH_2 [2]. The ubiquinol thus formed is released from the Q_B binding site into the membrane and replaced by an oxidized quinone from the pool of ubiquinone (Q) in the lipid membrane. The ubiquinol is then reoxidized by the cytochrome bc_1 complex, another membrane enzyme of the photosynthetic electron-transfer chain which functions as a ubiquinol oxidase/cytochrome reductase [3]. Water-soluble cytochrome *c* shuttles between these two complexes to close the electron transfer cycle. The cyclic mechanism of all these redox reactions acts as a light-driven proton pumping system, moving protons from the cytoplasm to the periplasm. The resultant H^+ gradient is the driving force for the synthesis of ATP.

Whereas the first (from Q_B to Q_B^-) and second (from Q_B^- to Q_BH_2) reduction steps have been largely investigated in bacterial RCs [4,5], the release of Q_BH_2 from its binding pocket towards the membrane and its replacement by an oxidized quinone from the Q pool is less characterized [6]. Even less characterized is the reduction of the Q pool in the native chromatophore membrane upon multiple turnovers of electron transfer and only indirect evidence for this process has been provided [7,8]. Vibrational infrared spectroscopy has proved to be a technique well-suited to investigate the structure and the interactions of quinones in various environments (solvent, lipid bilayer, protein) and in different redox states ([9–13] and references therein). Notably, semiquinone-minus-quinone Fourier transform infrared (FTIR) difference spectra have been obtained for the primary (Q_A) and the secondary (Q_B) quinone electron acceptors in RCs from the purple bacteria *Rhodobacter* (*Rb.*) *sphaeroides* and *Rhodopseudomonas viridis* (for a review see [9] and references therein). Light-induced formation of ubiquinol in situ lacks such a characterization. The possibility of using FTIR difference spectroscopy to investigate the molecular mechanisms accompanying QH_2 release, however, relies on the identification of marker bands for the target molecule. In the present work, steady-state and time-resolved FTIR difference spectroscopy have been applied to investigate the photoreduction of the pool of native ubiquinones in *Rb. sphaeroides* and to identify IR marker bands for QH_2 formation inside the photosynthetic membrane.

2. Materials and methods

Chromatophores from *Rb. sphaeroides* were prepared by French-press treatment as described in [14] and purified by sucrose-gradient. Samples for FTIR measurements were prepared by diluting chromatophores to a concentration of 3 μM in RCs in 70 mM Tris buffer

*Corresponding author. Fax: (33)-1-69088717.
E-mail address: nabedryk@dsvidf.cea.fr (E. Nabedryk).

[1] Present address: Instituto per la Ricerca Scientifica e Tecnologica, Via Sommarive 18, 38050 Povo (Trento), Italy.

Abbreviations: RC, reaction center; Q, quinone; QH_2, quinol; Q_n, 2,3-dimethoxy-5-methyl-6-polypropenyl-1,4-benzoquinone; Q_A and Q_B, primary and secondary quinone electron acceptors, respectively; P, primary electron donor; FTIR, Fourier transform infrared

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(pH 8) and adding 10 μM antimycin and 10 μM myxothiazol as specific inhibitors of the cytochrome bc_1 complex. To avoid contributions arising from the photo-oxidation of the primary donor P_{870}, 10 mM sodium ascorbate and 20 mM 2,3,5,6-tetramethyl-*p*-phenylenediamine (diaminodurene) were present as external redox components to ensure fast reduction of P^+_{870}. The suspension was centrifuged at 220 000 × *g* for 15 min and the obtained pellet was squeezed and sealed between two CaF_2 windows. Samples in ^2H_2O were prepared by diluting chromatophores in ^2H_2O and adding inhibitors and redox components in ^2H_2O. At least two centrifugations and resuspensions of the pellet in ^2H_2O were performed. This procedure leads to the deuteration of about 60% peptide NH groups of the proteins in the membrane.

A Bruker IFS88 FTIR spectrometer equipped with a photoconductive MCT-A detector and a KBr beam splitter was used. Time-resolved FTIR spectra were recorded under and after continuous illumination of the sample with a 250 W tungsten-halogen lamp, using rapid-scan conditions adapted from those described in [15]. Typically, an experimental cycle started with the recording and averaging of 200 reference interferograms in the dark (duration: 8 s). Then the light was switched on using a shutter and interferograms were recorded and averaged in groups of 10 (duration: 434 ms). After 4.3 s the light was switched off and interferograms were recorded and averaged in groups of 10 to monitor the 'relaxation' of the sample. FTIR difference spectra at various times under or after the continuous illumination were calculated from the single beam spectrum (corresponding to the Fourier transform of averaged interferograms) obtained in the dark before the onset of the light and the single beam spectrum obtained at time *t*. The results from ~700 cycles were averaged. Between cycles a delay time of a few minutes was set to allow a complete relaxation of the system. For steady-state experiments, difference spectra were calculated from interferograms recorded in a time window of 5.3 s before and after a sequence of multiple laser flashes (Nd:YAG laser, 7 ns, 530 nm, ~20 mJ, 5 Hz, Quantel) or a 4.3 s continuous illumination.

3. Results

Fig. 1 compares steady-state FTIR difference spectra obtained on chromatophores from *Rb. sphaeroides* after 10 (spectrum a) or 20 (spectrum b) saturating laser flashes, or immediately after continuous illumination (spectrum c). Under these conditions, where a fast donor/mediator couple is present, several Q molecules are expected to have undergone reduction for each RC. In these FTIR difference spectra, ubiquinone modes will contribute as negative bands while ubiquinol modes will appear as positive bands.

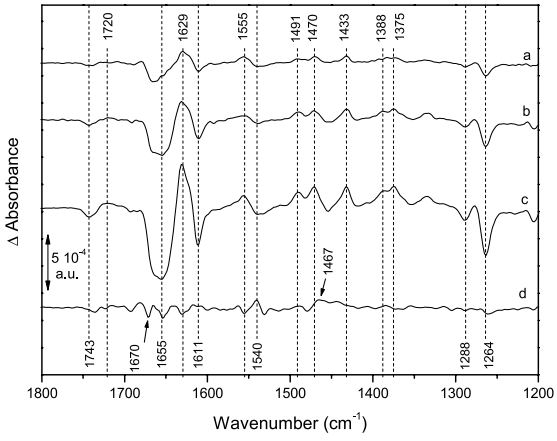

Fig. 1. Light-induced FTIR difference spectra of *Rb. sphaeroides* chromatophores recorded after 10 saturating flashes (trace a), 20 saturating flashes (trace b), after 3.5 s of continuous illumination (trace c), and after continuous illumination in the presence of terbutryn (trace d). Spectra are an average of two samples. Each trace corresponds to ~40 000 averaged interferograms. The frequency of the bands is given at ±1 cm^{-1}. Spectral resolution: 4 cm^{-1}. $T = 281 \pm 1$ K.

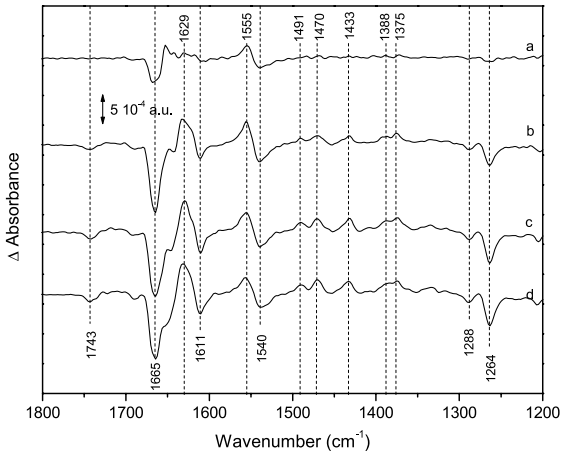

Fig. 2. Time evolution of the FTIR difference spectra of *Rb. sphaeroides* chromatophores after onset of continuous illumination. Trace a: recorded between 0 and 434 ms. Trace b: recorded between 1302 and 1736 ms. Trace c: recorded between 3010 and 3444 ms. Trace d: recorded between 3878 and 4312 ms. For each trace, 7000 interferograms were averaged.

Three negative bands at 1611, 1288 and 1264 cm^{-1} in the difference spectra (Fig. 1a–c) coincide, within 1–2 cm^{-1}, with the frequencies reported in the literature for isolated Q_{10} [10,11,16,17], and Q_{10} incorporated into phospholipid bilayers [12,13]. The bands at 1288 and 1264 cm^{-1} have been assigned to the C–O–CH_3 modes from the 2- and 3-methoxy groups, and the 1611 cm^{-1} negative band to C=C stretching vibrations of the ring ([16] and references therein). For isolated ubiquinones, the C=O stretching modes are found in the 1650–1665 cm^{-1} region. A broad negative band is indeed observed between 1670 and 1640 cm^{-1} (Fig. 1a–c). However, in this region, changes in the protein amide I modes [18] are likely to take place and probably account also for the positive band at 1629 cm^{-1}.

A clear evidence that the 1611, 1288 and 1264 cm^{-1} bands

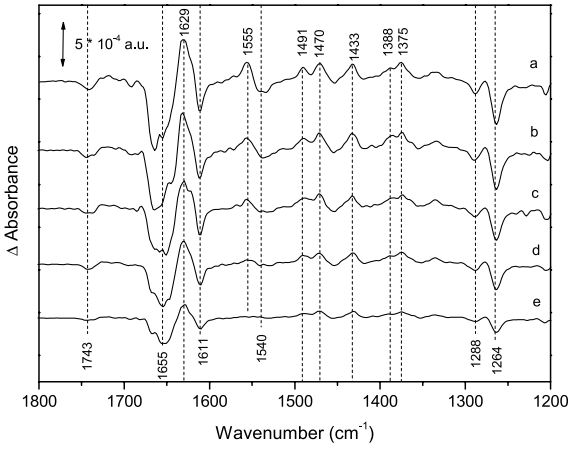

Fig. 3. Time evolution of the FTIR difference spectra of *Rb. sphaeroides* chromatophores after switching off the continuous illumination. Trace a: recorded between 0 and 434 ms. Trace b: recorded between 434 and 868 ms. Trace c: recorded between 1302 and 1736 ms. Trace d: recorded between 2200 and 3936 ms. Trace e: recorded between 5642 and 9982 ms. Traces a–d and e: 7000 and 70 000 averaged interferograms, respectively.

reflect light-induced reduction of several Q molecules per RC comes from the relationship between their intensities and the number of laser flashes fired. Due to the near-unity quantum yield of primary charge separation in the RC, one ubiquinone is expected to be reduced to ubiquinol per two laser flashes [2–5]. The spectrum recorded after 20 saturating flashes shows a two-fold increase in amplitude for the 1264, 1288 and 1611 cm^{-1} negative bands compared to the spectrum recorded after 10 saturating flashes (Fig. 1a,b). This demonstrates that, under the present experimental conditions (donor/mediator concentration, time between flashes), efficient photoreduction of the Q pool can be achieved. To estimate the maximum amplitude of the signals that can be generated, continuous illumination was applied (Fig. 1c). Under these conditions, the intensities of the 1264, 1288 and 1611 cm^{-1} negative bands are further increased, saturating at about twice the amplitudes induced by 20 laser flashes (Fig. 1b). These observations show that ~20 Q molecules can be photoreduced with saturating continuous illumination.

Ubiquinol vibrations should appear as positive bands in the FTIR difference spectra. Five main positive features at 1491, 1470, 1433, 1388 (shoulder) and 1375 cm^{-1} show intensities that are proportional to the number of laser flashes (Fig. 1a,b). These bands also increase after continuous illumination (Fig. 1c). Moreover, in all these spectra (Fig. 1a–c), the intensity of the positive bands is linearly correlated to the intensity

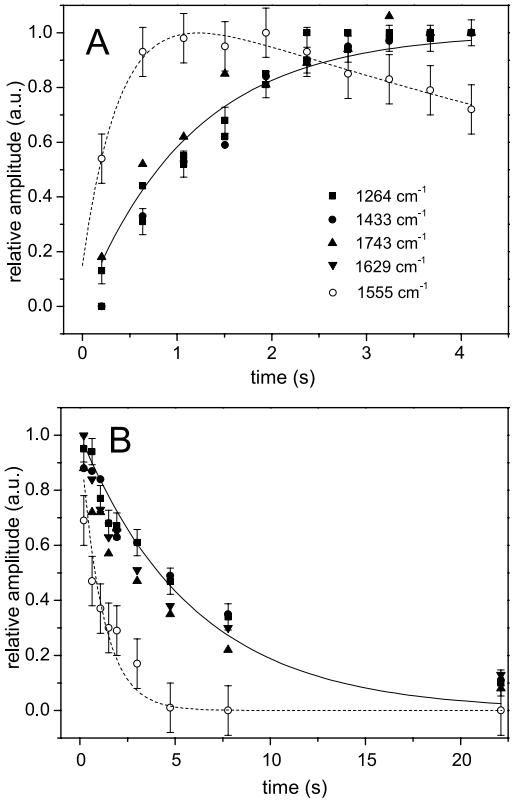

Fig. 4. Temporal evolution at several IR frequencies during continuous illumination (graph A) and after illumination (graph B) as calculated from Figs. 2 and 3, respectively. Bands were normalized to the maximum amplitude. Best-fitting curves as well as error bars are shown for the 1555 cm^{-1} (dashed line) and 1264 cm^{-1} (continuous line) bands. Single exponential functions were used as fitting curves except for the 1555 cm^{-1} band in graph A where a bi-exponential function was used to take into account the decay at long times.

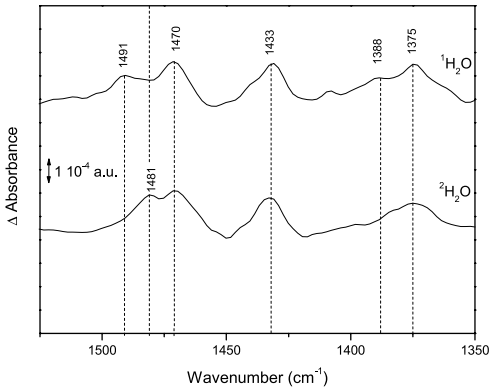

Fig. 5. Comparison of FTIR difference spectra of *Rb. sphaeroides* chromatophores obtained in ^1H_2O and ^2H_2O. Spectra were recorded during continuous illumination.

of the negative bands of ubiquinone at 1264, 1288 and 1611 cm^{-1}. Therefore, the positive bands at 1491, 1470, 1433, 1388 and 1375 cm^{-1} can be attributed to QH_2 formation.

The assignment of bands to Q and QH_2 presented above is strengthened by a control experiment performed with chromatophores in the presence of terbutryn, an inhibitor which binds to the Q_B site in the RC protein and therefore prevents ubiquinol formation. The corresponding FTIR difference spectrum obtained after saturating continuous illumination is shown in Fig. 1d. Comparison of the spectra in Fig. 1c (no inhibitor) and Fig. 1d (plus inhibitor) shows that all the negative and positive bands attributed to Q disappearance and QH_2 formation, respectively, are absent when terbutryn is bound (Fig. 1d). Instead, spectrum d in Fig. 1 reflects reduction of Q_A with characteristic peaks at 1670(−) and 1467(+) cm^{-1} [16,17,19]. It should also be noted that the characteristic positive band of Q_B^- at 1479 cm^{-1} [9] is absent in spectra displayed in Fig. 1a–c.

Fig. 2 shows time-resolved FTIR difference spectra of *Rb. sphaeroides* chromatophores under continuous illumination. The three negative bands due to disappearance of ubiquinone (1264, 1288 and 1611 cm^{-1}) and the five positive bands attributed to formation of QH_2 (1491, 1470, 1433, 1388 and 1375 cm^{-1}) grow simultaneously, showing the dynamics of QH_2 formation under illumination. The changes of amplitude at several IR frequencies as a function of time are reported in Fig. 4A. It is shown that, under illumination, both kinetics of the 1264 cm^{-1} (Q) and 1433 cm^{-1} (QH_2) bands rise with the same $\tau_{1/2}$ of about 1 s. Time-resolved FTIR difference spectra recorded after switching off the lamp are displayed in Fig. 3. The bands attributed to the process of QH_2 formation also decay in synchronicity, indicating reoxidation of QH_2 to Q by the redox buffer with a $\tau_{1/2}$ of about 5 s, as deduced from the kinetics of the 1264 and 1433 cm^{-1} bands shown in Fig. 4B.

Fig. 5 compares the light-induced FTIR difference spectra obtained during continuous illumination for chromatophores in ^1H_2O and ^2H_2O in the 1525–1350 cm^{-1} QH_2 spectral range. Bands of ubiquinone at 1611, 1288 and 1264 cm^{-1} are not affected by $^1H/^2H$ isotope exchange (data not shown). After deuterium exchange, positive bands corresponding to QH_2 formation are observed at 1481, 1470, 1433 and 1375 cm^{-1}. Comparison of the two spectra in Fig. 5 shows that the main QH_2 bands (at 1470, 1433 and 1375 cm^{-1}) are not sensitive to $^1H/^2H$ exchange while the band at 1491 cm^{-1} in ^1H_2O downshifts to 1481 cm^{-1} in ^2H_2O. The 1388 cm^{-1}

shoulder on the 1375 cm^{-1} band also appears affected by $^1H/^2H$ exchange but the extent of downshift is unclear.

A differential signal at 1555(+)/1540(−) cm^{-1} is observed in all the spectra displayed in Figs. 1a–c, 2a–d and 3a–d. Under illumination (Fig. 2a–d), the amplitude of this signal saturates earlier with illumination time ($\tau_{1/2} \sim 350$ ms, see Fig. 4A for the kinetics at 1555 cm^{-1}) than the amplitude of bands reflecting QH_2 formation. After illumination, the 1555 cm^{-1} signal decays significantly faster (with a $\tau_{1/2}$ of ~800 ms, see Fig. 4B) than the QH_2 bands ($\tau_{1/2} \sim 5$ s), and this feature is not seen at long times after switching off the continuous illumination (Fig. 3e). All these observations indicate that the 1555/1540 cm^{-1} signal is not directly correlated with the Q to QH_2 transition of the whole Q pool. This signal lies in the amide II spectral range [18], possibly reflecting a conformational change of the protein. The possibility that this conformational change is attributable to heating effects from the actinic light on the protein is unlikely since the 1555/1540 cm^{-1} signal is not seen in the spectrum obtained for chromatophores in the presence of terbutryn (Fig. 1d). Conformational changes might be related to the release of the first quinol molecule (Q_BH_2) from the RC Q_B pocket.

In the 1700–1620 cm^{-1} range of the spectra (Figs. 1a–c, 2b–d and 3a–e), large absorption changes are also observed, especially the positive one at 1629 cm^{-1}. This latter band appears and decays with a kinetics comparable to that of QH_2 bands (Fig. 4). This suggests a conformational change of the protein (backbone or side chain) linked to QH_2 formation. Moreover, the shape of the negative band in the 1670–1640 cm^{-1} range, which varies during the reduction (Fig. 2) and the reoxidation (Fig. 3) of the Q pool, probably reflects the existence of complex protein conformational changes. Such differences in the kinetics and saturation behavior of the IR signals in the amide I region will deserve further investigations.

4. Discussion

In this work, we have used FTIR spectroscopy to monitor the photoaccumulation of ubiquinol in chromatophore membranes from *Rb. sphaeroides*. In the FTIR difference spectra, contributions from Q/QH_2 vibrations as well as possible changes from the protein or the membrane lipids accompanying the process of QH_2 formation are expected. Identification of IR bands to specific molecular vibrations of Q and QH_2 in situ is based on the comparison with spectra of Q/QH_2 obtained in vitro and the effects of $^1H/^2H$ exchange.

Electrochemical studies of double reduction of ubiquinone-2 (Q_2) in aqueous solution have been reported [20]. Oxidized-minus-reduced FTIR difference spectra of Q_2 show Q bands at 1660–1650, 1612, 1288 and 1264 cm^{-1}, and QH_2 bands at 1490, 1470, 1432 and 1388 cm^{-1}. This latter pattern compares very well with the 1491, 1470, 1433 and 1388 cm^{-1} positive bands of the difference spectra of chromatophores (Figs. 1–3), demonstrating that these bands arise essentially from vibrations of ubiquinol. It should be noticed that comparable frequencies for bands assigned to ubiquinol modes have also been observed in electrochemically induced FTIR difference spectra of cytochrome bo_3 from *Escherichia coli* (at 1488, 1468, 1432 and 1390 cm^{-1}) [20] and cytochrome bc_1 from *Rb. capsulatus* (at 1488, 1464, 1428 and 1388 cm^{-1}) [21].

In the 1500–1300 cm^{-1} range, modes from quinonic ring bonds (C–C), as well as from CH_2 and CH_3 groups, are expected to contribute ([11,16] and references therein). In IR absorption spectra of isolated ubiquinones, bands at 1449–1436 cm^{-1} and at 1381 cm^{-1} have been previously attributed to δCH_2 and δCH_3 modes from the hydrocarbon chain and the 5-methyl substituent [11,16]. Upon site-specific ^{13}C-labeling of the $C_1=O$ or $C_4=O$ carbonyls of Q_2, electrochemically induced FTIR difference spectra showed QH_2 bands at 1484, 1464, 1426 and 1384–1380 cm^{-1} [20]. The main QH_2 bands are therefore downshifted by ~6 cm^{-1} upon specific ^{13}C-labeling of either the C_1- or the C_4-carbonyl of Q_2, which is consistent with their assignments to ring C–C and $\delta CH_2/\delta CH_3$ modes [20]. Some of these modes are likely to be coupled to the OH/C–OH vibrations of the QH_2. Here, $^1H/^2H$ exchange on chromatophores, which should result in the deuteration of the two hydroxyl groups of the QH_2, was performed to investigate such a coupling. Fig. 5 shows that the QH_2 band observed at 1491 cm^{-1} in ^1H_2O is downshifted by ~10 cm^{-1} in ^2H_2O. The 1491 cm^{-1} band can therefore be attributed to a ring C–C mode coupled to a C–OH mode. On the other hand, the 1470, 1433 and 1375 cm^{-1} ubiquinol bands observed in chromatophores and which are not sensitive to $^1H/^2H$ exchange (Fig. 5) are tentatively assigned to reorganization of ring C–C (at 1470 and 1433 cm^{-1}), and CH_2/CH_3 (at 1433 and 1375 cm^{-1}) modes upon Q reduction.

In addition, the light-induced FTIR difference spectra corresponding to QH_2 formation in chromatophore membranes all display a broad differential feature at ~1743(−)/1720(+) cm^{-1} (Figs. 1–3) which is not significantly affected upon $^1H/^2H$ exchange (data not shown). In this region, contributions can arise from C=O stretching modes of the ester from pigments in the RC [22], of protonated Glu and Asp residues [23], or of the phospholipid head groups [24]. The first possibility is unlikely, as the intensity of the 1743/1720 cm^{-1} feature increases proportionally to the number of flashes (Fig. 1). The 1743 cm^{-1} band appears and decays with kinetics comparable to QH_2 formation (Fig. 4). Furthermore, if the 1743/1720 cm^{-1} feature was due to (accessible) protonated Glu or Asp side chains, a significant (3–10 cm^{-1}) frequency downshift upon $^1H/^2H$ exchange would be expected [23]. Therefore, we favor the explanation that the 1743/1720 cm^{-1} signal reflects a perturbation of the C=O stretching mode of phospholipid head groups induced by a different interaction of the lipids with ubiquinol and ubiquinone.

5. Conclusion

This FTIR work supports the existence of a homogeneous Q pool inside the chromatophore membrane. The maximum number of QH_2 molecules formed is ~20 QH_2 per RC, in agreement with the Q/RC ratio (20–30) determined by Q extraction [25,26]. The detection of the Q pool by FTIR difference spectroscopy provides a new approach to study the supramolecular organization of the photosynthetic electron transport chain [27]. In particular, the role of the PufX protein in organizing the membrane protein complexes for efficient QH_2 diffusion and QH_2/Q exchange between the RC and cytochrome bc_1 [28,29] could be investigated.

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