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# Effects of dehydration on light-induced conformational changes in bacterial photosynthetic reaction centers probed by optical and differential FTIR spectroscopy

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ABSTRACT

Following light-induced electron transfer between the primary donor (P) and quinone acceptor ($Q_A$) the bacterial photosynthetic reaction center (RC) undergoes conformational relaxations which stabilize the primary charge separated state $P^+Q_A^-$. Dehydration of RCs from *Rhodobacter sphaeroides* hinders these conformational dynamics, leading to acceleration of $P^+Q_A^-$ recombination kinetics [Malferrari et al., J. Phys. Chem. B 115 (2011) 14732-14750]. To clarify the structural basis of the conformational relaxations and the involvement of bound water molecules, we analyzed light-induced $P^+Q_A^-/PQ_A$ difference FTIR spectra of RC films at two hydration levels (relative humidity $r=76\%$ and $r=11\%$). Dehydration reduced the amplitude of bands in the 3700–3550 $cm^{-1}$ region, attributed to water molecules hydrogen bonded to the RC, previously proposed to stabilize the charge separation by dielectric screening [Iwata et al., Biochemistry 48 (2009) 1220–1229]. Other features of the FTIR difference spectrum were affected by partial depletion of the hydration shell ($r=11\%$), including contributions from modes of P (9-keto groups), and from NH or OH stretching modes of amino acidic residues, absorbing in the 3550–3150 $cm^{-1}$ range, a region so far not examined in detail for bacterial RCs. To probe in parallel the effects of dehydration on the RC conformational relaxations, we analyzed by optical absorption spectroscopy the kinetics of $P^+Q_A^-$ recombination following the same photoexcitation used in FTIR measurements (20 s continuous illumination). The results suggest a correlation between the observed FTIR spectral changes and the conformational rearrangements which, in the hydrated system, strongly stabilize the $P^+Q_A^-$ charge separated state over the second time scale.



## 1. Introduction

The reaction center (RC) of purple photosynthetic bacteria is an intrinsic membrane pigment–protein complex which catalyzes the initial photochemical processes in the conversion of solar excitation energy into chemical free energy. The complex provides the scaffold to bind redox cofactors which perform light-induced electron transfer (ET), resulting in a transmembrane electric charge separation. The ET reactions within the RC have been extensively characterized, and are discussed in several reviews [1,2]. Briefly, in the RC from *Rhodobacter* (*Rb.*) *sphaeroides*, following absorption of a photon, a special pair (P) of bacteriochlorophyll molecules, located close to the periplasmic side of the complex, enters the first excited singlet state (P*) from which an electron is transferred, via an intermediate bacteriopheophytin cofactor, to a ubiquinone-10 molecule, acting as the primary ($Q_A$) acceptor on the cytoplasmic side of the RC complex. Following this primary charge separation, which leads in ~200 ps to the $P^+Q_A^-$ state, the electron is transferred from $Q_A^-$ to the secondary quinone acceptor, $Q_B$, on the 100-μs time scale. When RCs lack a quinone at the $Q_B$ site the electron on $Q_A^-$ recombines with the hole on $P^+$ with a room temperature rate constant $k$ ~10 $s^{-1}$ [1].

A large body of biochemical, spectroscopic and crystallographic data concurs to demonstrate that the RC undergoes conformational rearrangements upon light-induced charge separation [3–13]. The pivotal work of Kleinfeld and colleagues [4], subsequently revisited and extended by McMahon and coworkers [7], showed in particular that following a short (ns) photoexcitation the RC relaxes at room temperature from a dark-adapted to a light-adapted conformation, which stabilizes the primary charge separated state $P^+Q_A^-$. The two conformations can be trapped by freezing the RC at cryogenic temperatures in the dark and under illumination, respectively. Additionally,

*Abbreviations:* BChl, bacteriochlorophyll; FTIR, Fourier transform infrared; LDAO, lauryldimethylamine N-oxide; P, bacteriochlorophyll dimer; $Q_A$, primary quinone; $Q_B$, secondary quinone; *Rb.*, *Rhodobacter*; RC, reaction center

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at low temperature, the kinetics of $P^+Q_A^-$ recombination after an ns photoexcitation pulse becomes largely distributed in rate indicating that the RC is also trapped over a large ensemble of conformational substates, differing in the stability of the charge separated state. From these studies it was inferred that at room temperature, under physiological conditions, the RC protein rapidly solvates the altered electric charge distribution generated by a short photoexcitation, thus decreasing by about five times the rate of charge recombination. Notably, at room temperature the dielectric relaxation from the dark-adapted to the light-adapted conformation must occur over a time scale much shorter than that of $P^+Q_A^-$ recombination ($\tau \sim 10^{-1}$ s). The same holds for the interconversion between conformational substates which, at room temperature, averages the static heterogeneity of the RC observable only at cryogenic temperatures [7].

Besides these fast conformational dynamics, which is probed by the kinetics of $P^+Q_A^-$ recombination following a short photoexcitation in dark-adapted RCs, several observations indicate that, under continuous illumination, RCs undergo slow conformational rearrangements on the second to minute time scale [8,11,14,15]. The lifetime of charge recombination increases significantly upon lengthy photoexcitation suggesting that the persistence of the charge separated state drives additional structural rearrangements within the RC, which result in further stabilization of the charge separated states [11]. Elucidation of this non-equilibrium dynamic behavior is thought to be relevant when modeling the physiological functioning of the RC under fluctuating continuous illumination regimes.

Despite extensive efforts the structural bases of the conformational relaxations responsible for the short- and long-time stabilization of the primary charge separated state are still unclear. Spectroscopic and crystallographic studies have led to suggest different mechanisms, currently under lively debate, in which the prevailing role is played by: (a) alterations of the distance between the involved cofactors [4] and/or reorientation of $Q_A$ upon photoreduction [16], but recent results argue against these possibilities [17]; (b) small- and large-scale protein structural changes localized on the cytoplasmic side of the RC [5,9]; (c) proton uptake and fast protonation (on the 10–$10^2$ μs time scale) of aspartate or glutamate residues on the cytoplasmic side of the RC [18]; (d) rearrangements involving amino acid residues and bound water molecules near P [12,13]; (e) dielectric relaxation of water molecules weakly bound to the RC in the vicinity of the $Q_A$ quinone acceptor [9,19].

We have shown that dehydration of the RC deeply hampers the conformational RC relaxation following a short photoexcitation. Inhibition of the dynamics coupled to stabilization of the charge separated $P^+Q_A^-$ state can be achieved in dehydrated systems at room temperature both in the absence [20] and in the presence of a sugar glassy matrix embedding the RC [21–25]. In RC–detergent films, obtained in the absence of sugars, the hydration level of the RC can be accurately controlled by an isopiestic method and finely tunes the recombination kinetics of the $P^+Q_A^-$ state after a laser pulse, which, below a threshold value, becomes strongly accelerated and distributed in rate, mimicking at room temperature the effects observed at cryogenic temperature in water–glycerol glasses [20]. A thermodynamic and spectral analysis of the residual water bound to the RC–detergent complex led us to conclude that water interacting with the RC complex plays a critical role in controlling the transition from the dark- to the light-adapted conformations stabilizing the $P^+Q_A^-$ charge separated state [20]. The controlled dehydration of RC–detergent films represents therefore an attractive approach to modulate the RC dynamics and to gain insight into the mechanisms of light-induced conformational changes at room temperature.

In the present paper this approach has been combined with light-minus-dark FTIR difference spectroscopy, which proved to be a powerful method to characterize light-induced RC structural changes involving cofactors, protein residues, as well as water molecules interacting with the RC complex (see for instance [19,26–29]). Most of FTIR difference spectroscopy studies have been performed on well-hydrated RCs and the effects of dehydration on the light-induced FTIR difference spectra have not been examined systematically. We have chosen to analyze $P^+Q_A^-/PQ_A$ FTIR difference spectra in RC-LDAO complexes at two representative hydration levels, i.e. equilibrated at two values of relative humidity, $r = 76\%$ and $r = 11\%$, which correspond to a number of residual water molecules adsorbed to the RC–detergent complex differing by almost one order of magnitude [20]. As previously shown, dehydration from $r = 76\%$ to $r = 11\%$ removes the weakly bound water and even starts to deplete the tightly bound hydration shell of the protein, retarding markedly the RC conformational dynamics which stabilizes the $P^+Q_A^-$ state formed by a short laser flash [20].

In the present work we have found that such dehydration affects significantly the light-minus-dark FTIR difference spectrum in the 4000–1200 $cm^{-1}$ range, altering different bands attributed to bound water, to protein residues, and to groups of the bacteriochlorophyll special pair P. To probe in parallel the effects of dehydration on the RC relaxation from dark-adapted to light-adapted conformations, we have analyzed by time-resolved optical absorption spectroscopy the kinetics of $P^+Q_A^-$ recombination following the same photoexcitation regime used in the FTIR measurements. The results point to a correlation between the observed FTIR spectral changes and the slow conformational rearrangements which strongly stabilize the $P^+Q_A^-$ charge separated state on a time scale of seconds.

## 2. Materials and methods

### 2.1. RC purification and preparation of dehydrated RC-LDAO films

RCs were purified from *Rb. sphaeroides wt 2.4.1* strain as described previously [30], using lauryldimethylamine N-oxide (LDAO) as a detergent. The occupancy of the $Q_B$ site was estimated to be approximately 57% from the relative amplitude of the slow kinetic phase ($\tau \approx 1.8$ s) of $P^+$ decay after a laser pulse [31] measured by kinetic spectrophotometry at 422 nm in solution samples (see Section 2.3).

RC-LDAO films were prepared on $CaF_2$ windows as described previously [20] using 40–60 μL aliquots of a solution containing 60 μM RC, 10 mM TRIS HCl, pH 8.0, 0.025% LDAO and 10 mM o-phenantroline. We used o-phenanthroline at this high concentration to fully inhibit the electron transfer from $Q_A^-$ to $Q_B$, in order to obtain a pure $P^+Q_A^-/PQ_A$ FTIR difference spectrum (see Section 3.3). Since at high concentrations o-phenanthroline may partially displace also $Q_A$ from its binding site, thus inhibiting primary photochemistry in a fraction of RCs [32], we checked the functionality of the $Q_A$ site. We found that the addition of 10 mM o-phenanthroline did not affect significantly the extent of flash-induced $P^+Q_A^-$ charge separation both in RC solution and in dehydrated RC films. A small gastight compartment of volume ≈ 1 mL, was obtained by placing a second $CaF_2$ window onto the one carrying the RC–detergent film in a clipping sample holder, using a rubber O-ring covered with vacuum grease, interposed between the two windows, as a spacer.

The relative humidity $r$ within the compartment containing the sample was controlled by a few microliter drops of saturated NaCl or LiCl solutions, to achieve values of relative humidity at 281 K of 76% and 11% respectively [20]. The hydration level of the samples at equilibrium was evaluated from FTIR spectra recorded between 6000 and 1000 $cm^{-1}$, using the area of the ($\nu_2 + \nu_3$) combination band of water (peaking at ≈5180 $cm^{-1}$) and the area of the amide II band of the RC (at ≈1550 $cm^{-1}$) as an internal standard [20]. These determinations yielded ($H_2O$/RC) molar ratios of $1.1 \times 10^3$ and $8.2 \times 10^3$ in RC films equilibrated at $r = 11\%$ and 76% respectively, in agreement with previous results [20].

RC-LDAO equilibrated at $r = 76\%$ with $D_2O$ or $H_2^{18}O$ were obtained following a sequence of dehydration/re-hydration equilibria [33]. Essentially the film was first dehydrated by equilibrating it, within a gastight chamber of volume ≈40 mL, with about 3 mL of a saturated

LiCl solution in $H_2O$ for 3 hours. The film was then rehydrated in a $D_2O$ or $H_2^{18}O$ atmosphere by replacing in the same chamber the $H_2O$ lithium chloride solution with a saturated NaCl solution in $D_2O$ or $H_2^{18}O$. The efficiency of the isotope substitution procedure has been evaluated in the range between 90% and 100% from the residual differential bands attributed to $H_2O$, detectable in $P^+Q_A^-/PQ_A$ spectra of RC-LDAO films re-hydrated at $r=76\%$ with $D_2O$ and $H_2^{18}O$. In the case of $D_2O$ substitution, an extent of isotopic replacement larger than 95% has been independently estimated from the area of the residual $(\nu_2+\nu_3)$ combination band of $H_2O$ in FTIR spectra recorded in the dark-adapted RC-LDAO film [33].

### 2.2. FTIR spectroscopic measurements

FTIR spectra were recorded on a Bruker IFS 88 spectrometer, equipped with MCT-A and DTGS detectors. The sample was kept is a nitrogen cryostat (Oxford instruments) to keep the temperature stable within ±1 °C during the measurements. For the evaluation of the hydration level of the RC films an absorbance spectrum (obtained by averaging 500 interferograms), was recorded in the dark over the 6000–1000 $cm^{-1}$ range using the DTGS detector.

Light-minus-dark FTIR difference spectra were recorded following essentially the procedure described by Breton et al. [34]. Interferograms in the 4000–1200 $cm^{-1}$ range were acquired at 281 K before and during continuous illumination (lasting 20 s) using a MCT-A detector.[1] The mirror speed was set to 2,531 cm $s^{-1}$, and the interferogram were collected in the double-sided, forward-backward mode. The duration of photoexcitation was controlled by a Uniblitz electroprogrammable shutter system (Vincent Associates, Rochester, NY) characterized by a closure time of 3 ms.

The results from several cycles (obtained on at least two different samples) were averaged to improve the signal-to-noise ratio (for the exact number of averaged interferograms, see later). Between cycles an appropriate delay time (80 s and 140 s for the RC films equilibrated at $r=11\%$ and $r=76\%$, respectively) allowed a complete recovery of the RC neutral state after the light-induced charge separation (the delay time was set looking at the kinetics of $P^+Q_A^-$ recombination monitored by time-resolved visible absorption spectroscopy following a 20 s period of continuous photoexcitation, see Results, Section 3.5).

Continuous illumination was provided by a 250 W tungsten halogen lamp (Oriel) collimated by an optical condenser and filtered through a water layer 8 cm thick, a 50% neutral density filter and two coloured glass filters, resulting in a transmitted band centred at 760 nm with 0.01 transmittance for $\lambda<700$ nm and $\lambda>850$ nm.

$P^+Q_A^-/PQ_A$ FTIR difference spectra at $r=76\%$ and $r=11\%$ in $H_2O$ were obtained by averaging 1.300.000 and 2.280.000 interferograms, respectively; $P^+Q_A^-/PQ_A$ FTIR difference spectra at $r=76\%$ in $D_2O$ and $H_2^{18}O$ were obtained by averaging 396.000 and 270.000 interferograms, respectively.

### 2.3. Time-resolved optical absorption spectroscopy

The kinetics of $P^+Q_A^-$ recombination after a laser pulse or following a 20 s period of continuous photoexcitation were measured at 422 nm and 450 nm [35], using a kinetic spectrophotometer of local design [23,36]. Flash photoexcitation was provided by a frequency doubled Nd:YAG laser (Quanta System, Handy 710) delivering 200 mJ pulses of 7 ns width. Continuous photoexcitation was accomplished by using the same illuminator and shutter system employed for the light-minus-dark FTIR spectroscopic measurements, except that the two optical filters were replaced with a coloured glass long-pass filter with a cut-on wavelength of 780 nm.

Kinetic traces of $P^+Q_A^-$ recombination are the average of several (between 4 and 12) signals, depending on the signal to noise ratio. During averaging the sample was allowed to dark-adapt between successive photoexcitations for the same time period used in the FTIR light-minus-dark differential measurements (see Section 2.2). $P^+Q_A^-$ kinetics were fitted to a single power law or to the sum of two power law (see Section 3.5), by using non-linear least-square minimization routines based on a modified Marquardt algorithm [37]. Confidence intervals for the fitting parameters were evaluated numerically through an exhaustive search method, as described in detail by Francia et al. [24].

## 3. Results

Previous studies (reviewed in [26]; see also [38,39]) have shown that the $P^+Q_A^-$ light-minus-dark FTIR difference spectrum in the 4000–1000 $cm^{-1}$ range contain a large number of bands, attributed to the photooxidation of the primary donor, to $Q_A$ reduction, and to vibrational modes reflecting the protein response to the formation of $P^+Q_A^-$ state. Dehydration of the RC films (from $r=76\%$ to $r=11\%$) induces a number of changes in the $P^+Q_A^-/PQ_A$ FTIR difference spectrum, which can be conveniently grouped according to their spectral region and are examined in detail in the following paragraphs.

### 3.1. The $P^+$ electronic band region (3150–1900 $cm^{-1}$)

Over the 4000–1000 $cm^{-1}$ spectral range the largest contribution to the differential light-minus-dark spectrum is given by a broad band centred around 2600 $cm^{-1}$ which is due to an electronic transition of $P^+$ [40]. In RC-LDAO films equilibrated at $r=76\%$ the amplitude of the electronic band of $P^+$ is systematically larger than in films equilibrated at $r=11\%$ (not shown). In view of the comparable RC concentration and optical path of the samples, as evaluated from the area of the RC amide II band measured in the dark, the smaller amplitude of the light-induced band in the dry film implies that the extent of P steadily oxidized under continuous photo-excitation is systematically lowered upon decreasing the hydration of the system. This observation was confirmed by optical spectroscopy performed in parallel to the FTIR measurements (see Section 3.5) which also indicated a much lower P photooxidation level under continuous illumination (cf. Fig. 5A). This behavior is consistent with the strong acceleration of flash induced $P^+Q_A^-$ charge recombination kinetics observed in RC-LDAO films upon dehydration (see Section 3.5 and Fig. 5C). We expect, in fact, that the steady level of photo-oxidized P is determined by the competition between light-induced charge separation and $P^+Q_A^-$ recombination, and, therefore, that the faster charge recombination in the dehydrated system results in a decreased amplitude of the electronic band of $P^+$.

As a consequence, to better compare the $P^+Q_A^-/PQ_A$ FTIR difference spectra measured at the two hydration levels, we have normalized them to the extent of photo-induced $P^+$, estimated from the amplitude of the differential bands at 1749/1739 $cm^{-1}$ attributed to the 10*a*-ester C=O mode of the bacteriochlorophylls of the P special pair [26].

On the top and on the lower wavenumber ridge of the electronic band in the 2900–2200 $cm^{-1}$ interval, a series of 6 smaller positive broad bands can be identified (Fig. 1A). Although over this wavenumber range the electronic $P^+/P$ band largely dominates the difference spectrum, 4 positive peaks can be resolved at 2818, 2756, 2709, and 2611 $cm^{-1}$ which appear equivalent to corresponding bands detected in $Q_A^-/Q_A$ spectra [19]. Our $P^+Q_A^-/PQ_A$ FTIR difference spectra include additional positive peaks at 2584 and 2505 $cm^{-1}$ (see Fig. 1A). The bands detected in the $Q_A^-/Q_A$ FTIR difference spectrum were tentatively assigned to the strongly hydrogen bonded N—H group of His-M219 [19,41], which is part of the so-called ($Q_A$–HisM219–Fe–HisL190–$Q_B$)

[1] Light-induced FTIR difference spectra were also recorded for samples at the two hydration levels using a DTGS detector, in order to assess possible spectral distortions in the experiments using the MCT-A detector. The difference spectra resulted identical to those reported in Figs. 1–4, even though with a worse signal-to-noise ratio.

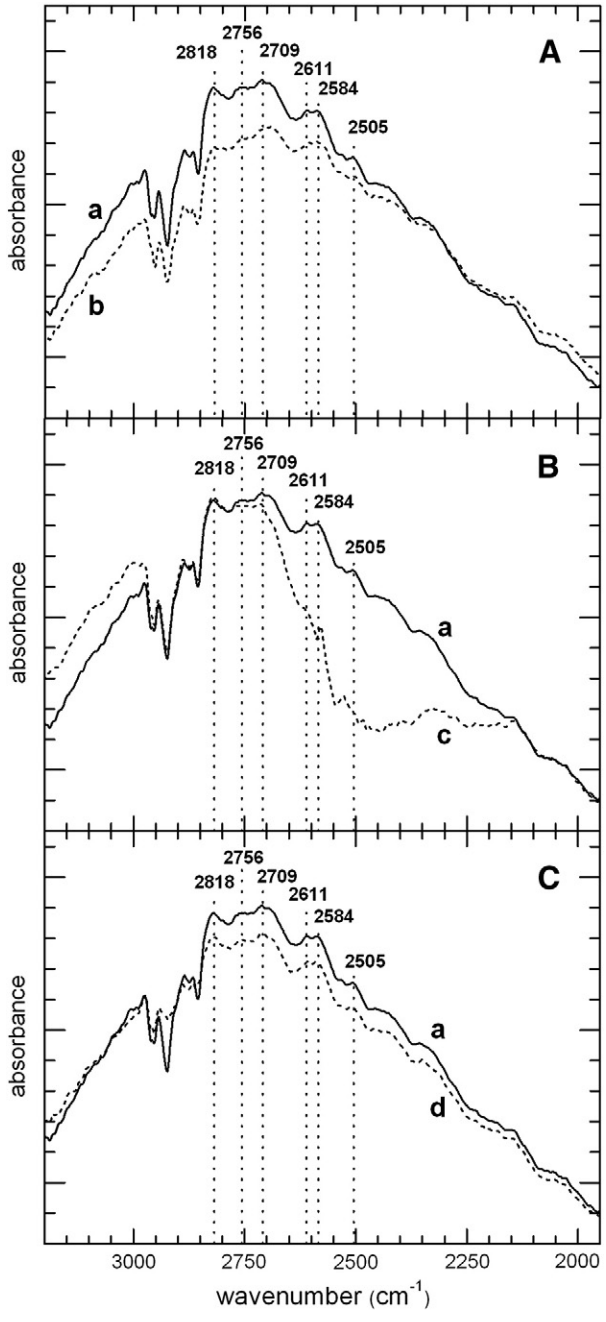


**Fig. 1.** Light-induced $P^+Q_A^-/PQ_A$ difference FTIR spectra of RC-LDAO films in the 3200–2000 $cm^{-1}$ region. Panel A compares the spectra recorded in films hydrated with $H_2O$ at $r = 76\%$ (continuous line, *a*) and $r = 11\%$ (dashed line, *b*). The effects of $D_2O$ and $H_2^{18}O$ substitution at $r = 76\%$ are shown in panel B (dashed line, *c*) and C (dashed line, *d*), respectively. Peaks identified in the $H_2O$ spectrum at $r = 76\%$ are marked by dotted vertical lines. The increment between major ticks of the *y*-axis corresponds to $3 \times 10^{-4}$ absorbance units. The spectra have been normalized on the basis of the differential bands at 1749/1739 $cm^{-1}$ attributed to the 10*a*-ester C=O mode of P.

iron–histidine complex. Conversely, contributions from protonated water molecules, as proposed previously [42], are unlikely, as no spectral shifts upon $H_2^{18}O/H_2O$ exchange are observed (see later; see also [19]). Interestingly, bands at nearly the same position were identified in $Q_A^-/Q_A$ FTIR difference spectra of photosystem II and attributed to Fermi resonance of harmonics or combination bands of imidazole ring modes with the hydrogen bonding NH stretching vibration [43]; a tentative assignment to the same physical phenomenon can be made on the basis of the similarity of the ($Q_A$–His–Fe–His–$Q_B$) protein motif between the bacterial RC and Photosystem II [44]. Such assignment is relevant to investigation of the working mechanism of the bacterial RC, as a recent time-resolved FTIR study suggests implication of the iron–histidine complex in the events associated with the $Q_A^-Q_B \rightarrow Q_AQ_B^-$ ET reaction [45].

In line with this tentative attribution, in our $P^+Q_A^-/PQ_A$ spectra equilibrated at $r = 76\%$, $D_2O$ substitution causes a strong decrease or elimination of the bands (Fig. 1B), as already reported for $Q_A^-/Q_A$ difference spectra [42], except for the band at 2818 $cm^{-1}$ (that remains unchanged) and for the bands at 2756 and 2709 $cm^{-1}$ (the intensity and shape of which seem however slightly altered), while $H_2^{18}O$ replacement does not appear to cause any shift in the band peaks (Fig. 1C), in agreement with what observed by Iwata and colleagues [19] in the $Q_A^-/Q_A$ difference spectrum. The position and the amplitude of these bands do not change significantly when the films are equilibrated at a lower relative humidity, $r = 11\%$ (Fig. 1A). This is also in agreement with the absence of spectral contributions from water molecules to these bands.

The fact that shape and peak wavenumber of the 2818, 2756, 2709, 2611, 2584 and 2505 $cm^{-1}$ positive bands are not significantly affected by a strong dehydration of the sample means that the molecular group(s) responsible of these bands (for the 2818, 2756, 2709, 2611 $cm^{-1}$ bands probably the His M219 side chain) are not sensitive to the dehydration of the RC, in contrast to behavior of other molecular groups (see Sections 3.2–3.4). It has to be noticed, however, that the broad band to which the much smaller bands mentioned above are overlaid, does not coincide in the "hydrated" and in the "dehydrated" sample. Since the two spectra of Fig. 1A have been normalized on the basis of the differential bands at 1749/1739 $cm^{-1}$ attributed to the 10*a*-ester C=O mode of P, the difference between the spectra appears to reflect a significant alteration of the electronic band of $P^+$ induced by dehydration. Further experiments are required to better clarify the issue.

### *3.2. $P^+Q_A^-/PQ_A$ light-minus-dark FTIR difference spectra in the 3750–3550 $cm^{-1}$ range*

The $P^+Q_A^-/PQ_A$ difference spectrum of RC-LDAO films hydrated at $r = 76\%$ with $H_2O$ is shown in Fig. 2 (curve *a*) in the spectral range where OH vibrations due to weakly hydrogen bonded water molecules are expected to contribute [19,46–48]. In spite of the weakness of the signals, two positive peaks and one negative peak can be identified: the two positive bands are centered at 3664 $cm^{-1}$ and at ~3628 $cm^{-1}$ (broad) and the negative band peaks around 3587 $cm^{-1}$. Although this region should be free of spectral contribution other than those mentioned above, in order to safely assign these bands to weakly hydrogen bonded water molecules, $P^+Q_A^-/PQ_A$ spectra were measured in RC-LDAO films hydrated at $r = 76\%$ alternatively in $D_2O$ and $H_2^{18}O$ (see Section 2.1). Upon substitution of water with $D_2O$ and $H_2^{18}O$ the bands due to water molecules are expected to undergo a red-shift of about 900 $cm^{-1}$ and ≈6–18 $cm^{-1}$, respectively [19,48]. In principle the substitution of $H_2O$ with $H_2^{18}O$ provides by itself a definitive test for the assignment of these bands to water molecules. The $D_2O$ substitution, causing a much larger bandshift, allows additionally a better estimate of the efficiency of the isotopic replacement procedure.

$P^+Q_A^-/PQ_A$ spectra of RC-LDAO films hydrated with $D_2O$ and $H_2^{18}O$, compared to the one obtained in RC-LDAO films equilibrated at the same relative humidity ($r = 76\%$) in $H_2O$, are presented respectively in Fig. 2A (curve *b*) and 2B (curve *c*). As a consequence of $D_2O$ substitution the three peaks identified above completely disappear. In the $H_2^{18}O$ substituted sample the peaks detected at 3664 and 3628 $cm^{-1}$ in the $H_2O$ hydrated sample downshift to 3652 and 3617 $cm^{-1}$, respectively, whereas the negative peak observed in $H_2O$ at 3587 $cm^{-1}$ disappears. The complete removal of the bands from the 3750–3550 $cm^{-1}$ spectral range after $D_2O$ substitution and the 12 and 11 $cm^{-1}$ shifts of the peaks at 3664 and 3628 $cm^{-1}$ observed upon $H_2^{18}O$ substitution are fully consistent with the attribution of these two latter differential bands to

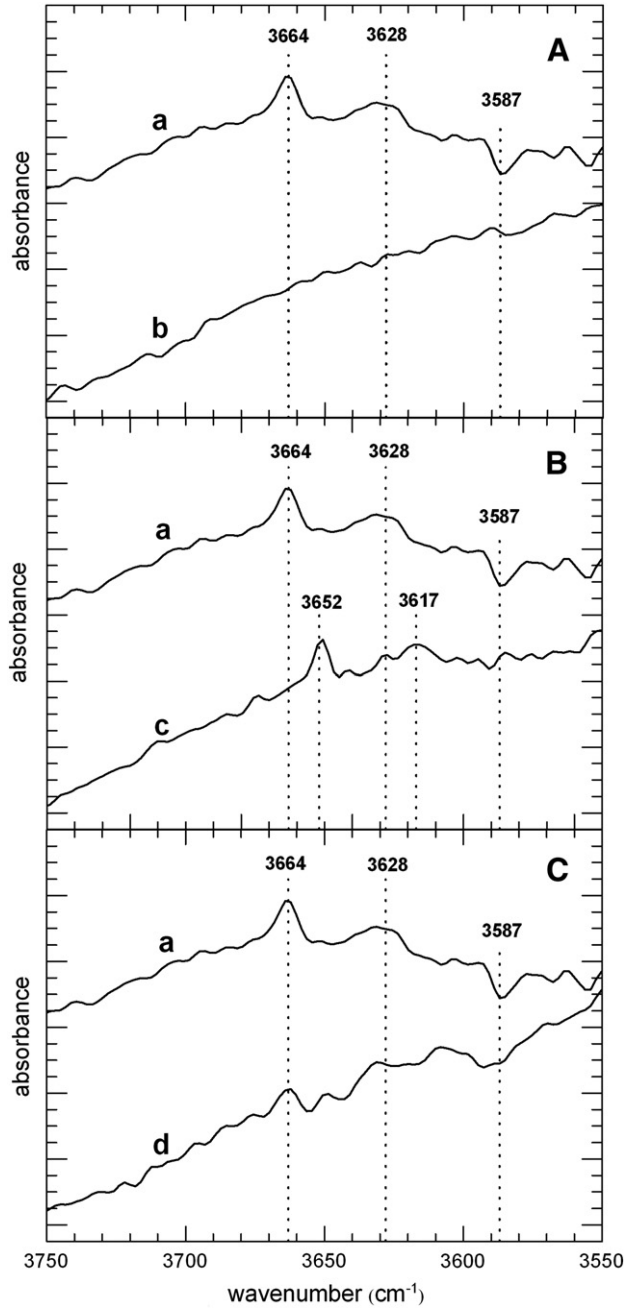


**Fig. 2.** Light induced $P^+Q_A^-/PQ_A$ FTIR difference spectra of RC-LDAO films in the 3750–3550 $cm^{-1}$ range. The spectrum recorded in films hydrated with $H_2O$ at $r=76\%$ (curve *a*) is compared with those measured following $D_2O$ (panel A, curve *b*) and $H_2^{18}O$ substitution (panel B, curve *c*). Panel C compares the spectrum of RC-LDAO films equilibrated with $H_2O$ vapor at $r=76\%$ (curve *a*) and $r=11\%$ (curve *d*). The increment between major ticks of the y-axis corresponds to $1\times10^{-5}$ absorbance units; spectra have been offset for visual clarity.

weakly hydrogen bonded water molecules. In the $H_2^{18}O$ hydrated samples, a small positive peak seems to be still present at 3628 $cm^{-1}$, but we are reluctant to interpret it as a residual of the band observed at this wavenumber in $H_2O$ hydrated samples, being its amplitude barely above the noise level. As mentioned above, the expected shift of the 3587 $cm^{-1}$ negative band could not be detected in the $H_2^{18}O$ hydrated samples, possibly due to interference with other vibrational bands. However, since a similar band, centered at 3587 $cm^{-1}$ and undergoing the expected shift in $H_2^{18}O$, has been observed in $Q_A^-/Q_A$ spectra [19], we tentatively assign the band observed by us at the same wavenumber also to weakly H-bonded water molecules.

Fig. 2C compares the spectrum of the hydrated RC-LDAO film, equilibrated with water vapour at $r=76\%$ (curve *a*), with that of the partially dehydrated sample, equilibrated at $r=11\%$ in $H_2O$ (curve *d*). As stated in Section 3.1, spectra have been normalized to the extent of photo-induced $P^+$. Due to the smaller extent of steadily photoxidized RC in the dehydrated sample the signal to noise ratio is worse for the film equilibrated at $r=11\%$. In spite of this, it is clear that the three bands observed at 3664, 3628 and 3587 $cm^{-1}$ in the hydrated sample are strongly reduced upon dehydration at $r=11\%$. We infer that, upon reducing the hydration of the RC-LDAO complex, the water molecules weakly hydrogen bonded to the RC which respond to the light-induced charge separation are either removed or unable to undergo the structural relaxations which give rise to the spectral changes observed in the hydrated samples.

### 3.3. $P^+Q_A^-/PQ_A$ light-minus-dark FTIR difference spectra in the 1900–1200 $cm^{-1}$ range

Fig. 3 shows the $P^+Q_A^-/PQ_A$ difference spectra recorded between 1800 and 1200 $cm^{-1}$ in RC-LDAO hydrated films, equilibrated with $H_2O$ vapour at $r=76\%$ and $r=11\%$. The two spectra are very similar, the differences lying mainly in the 1710–1450 $cm^{-1}$ region.

For a comprehensive description of the spectral region and a discussion of band attribution, the reader is referred to [26]. In the following we will discuss only the bands which are sensitive to the dehydration level of the RC. The bands associated with P photo-oxidation dominate the whole 1800–1200 $cm^{-1}$ region [26]. The positive peaks at 1716 and 1701 $cm^{-1}$ are given by the 9-keto C=O stretching modes of the two bacteriochlorophyll molecules $P_L$ and $P_M$ [26] of the oxidized primary donor P, respectively. Interestingly, the 1701 $cm^{-1}$ band upshifts to 1703 $cm^{-1}$ in the spectrum recorded at $r=11\%$, meaning that the stretching of the 9-keto of $P^+_M$ is sensitive to the hydration state of the sample. This slight upshift could mean that the interaction of $P^+_M$ with its surrounding environment is (slightly) modified by the hydration state of the sample. The observed modification of the intensity ratio between the 1716 $cm^{-1}$ band and the 1703 (1701) $cm^{-1}$ bands upon change of the relative humidity would agree with such hypothesis.

The presence in the light-induced spectrum of RC-LDAO films equilibrated with $H_2O$ vapour at $r=76\%$ of four negative peaks, at 1669, 1651, 1633 and 1603 $cm^{-1}$ (Fig. 3), is consistent with the assumption that, due to the presence of o-phenanthroline, we are observing a pure $P^+Q_A^-/PQ_A$ FTIR difference spectrum. In fact these four

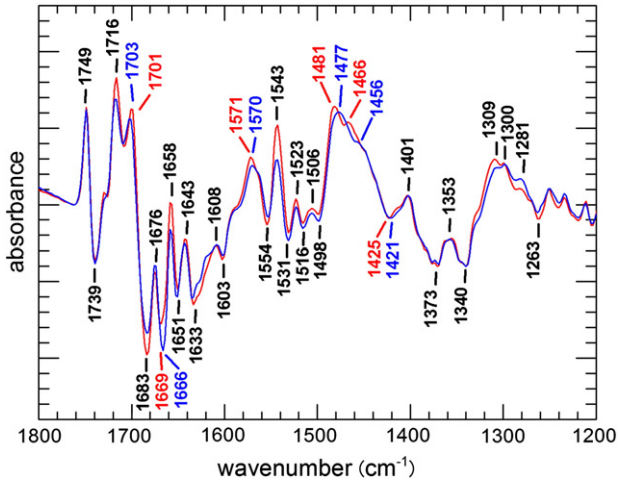


**Fig. 3.** Light induced $P^+Q_A^-/PQ_A$ FTIR difference spectra in the 1800–1200 $cm^{-1}$ range of RC-LDAO films equilibrated at a relative humidity $r=76\%$ (red line) and $r=11\%$ (blue line) in the presence of $H_2O$ vapor. The increment between major ticks of the *y*-axis corresponds to $1\times10^{-5}$ absorbance units.

peaks are a signature of the $P^+Q_A^-/PQ_A$ difference spectrum [5,49]. We conclude therefore that, even following continuous 20 s photoexcitations, o-phenanthroline fully inhibits ET from $Q_A^-$ to $Q_B$. This is also consistent with the spectrum recorded in the OH vibration region of weakly hydrogen bonded water, 3750–3550 $cm^{-1}$ (see Fig. 2), which is very similar to the $Q_A^-/Q_A$ spectrum and quite distinct from the $Q_B^-/Q_B$ spectrum [19]. The effective block of $Q_A^-$ to $Q_B$ ET will be relevant when interpreting the kinetics of charge recombination after a 20 s period of continuous illumination, i.e. under the same photoexcitation conditions used in the acquisition of differential FTIR spectra (see Section 3.5 and Discussion).

The whole 1670–1600 $cm^{-1}$ spectral region (where the amide I absorbs) is sensitive to the hydration state of the sample. In particular, the 1669 $cm^{-1}$ negative band observed at $r=76\%$ downshifts to 1666 $cm^{-1}$ when $r=11\%$. Unfortunately, no definitive assignment for this band has been made, so it is difficult to give a precise structural meaning to this shift. Other spectral changes upon dehydration to $r=11\%$ include the change in relative intensity of the bands between 1670 and 1600 $cm^{-1}$, suggesting that the hydration state of the RC influences the protein response to the $P^+Q_A^-$ charge separation.

At lower wavenumbers, a shift of the peaks at 1481 $cm^{-1}$ and 1466 $cm^{-1}$ at $r=76\%$ to 1477 $cm^{-1}$ and 1456 $cm^{-1}$, respectively, at $r=11\%$ can be observed. Slight modifications in the amplitude of the differential IR bands are observed in the whole 1800–1200 $cm^{-1}$ region (see for instance the decreased amplitude of the 1543 $cm^{-1}$ positive band in the $r=11\%$ spectrum). A detailed molecular interpretation is not, for the time being, possible; most probably these bands contain contributions from several vibrational modes. They can however be taken as a clear sign of the influence of the hydration state on the conformational rearrangements of the RC induced by $P^+Q_A^-$ charge separation.

### 3.4. $P^+Q_A^-/PQ_A$ light-minus-dark FTIR difference spectra in the 3550–3150 $cm^{-1}$ range

Between 3550 and 3150 $cm^{-1}$ the spectra of RC–detergent films contain contributions arising from the stretching of OH groups of strongly hydrogen bonded water molecules or of lateral groups of amino acids, as serine, tyrosine and protonated carboxylic acids [46,50], and from the stretching of hydrogen bonded NH groups of the protein [51]. Although this spectral region has been extensively studied in bacteriorhodopsin [46] and in the photoactive yellow protein from *Ectothiorhodospira halophyla* [52], as well as in photosystem II [53] a systematic and detailed analysis of FTIR difference spectra in this interval is still lacking in bacterial RCs.[2]

Fig. 4A shows the $P^+Q_A^-/PQ_A$ FTIR difference spectrum of hydrated RC-LDAO films, equilibrated with $H_2O$ at $r=76\%$. We also report the difference spectra recorded in RC-LDAO samples hydrated with $D_2O$ (Fig. 4C) or $H_2^{18}O$ (Fig. 4D) at $r=76\%$. The comparison with the spectra obtained after $D_2O$ and $H_2^{18}O$ substitution indicates that none of the many positive and negative bands detected in this spectral interval can be assigned to water molecules. In fact, no band disappears after $D_2O$ substitution (Fig. 4C), with the exception of the one peaking at 3483 $cm^{-1}$ in the $H_2O$ hydrated sample; however, after $H_2^{18}O$ substitution, no clear shift of this band is observed. This behavior suggests that the peak at 3483 $cm^{-1}$ is due to a NH or OH group of the peptide which undergoes deuterium exchange in $D_2O$. This agrees with the attribution suggested by Iwata et al. [19] for the positive peak detected at 3487 $cm^{-1}$ in the $Q_A^-/Q_A$ differential spectrum of fully hydrated RC films. Interestingly this peak, observed at 3487 $cm^{-1}$ in films equilibrated at $r=98\%$ [19], shifts to 3483 $cm^{-1}$ at $r=76\%$ and to 3481 $cm^{-1}$ at $r=11\%$ (Fig. 4B). Similarly the negative peak detected at 3501 $cm^{-1}$ in the $Q_A^-/Q_A$ spectrum at $r=98\%$ [19] appears to be shifted to 3498 $cm^{-1}$ at $r=76\%$ and to 3496 $cm^{-1}$ at $r=11\%$ in the $P^+Q_A^-/PQ_A$ difference spectrum. This behavior suggests a systematic shift of the negative and positive bands to lower wavenumbers upon dehydration of the RC complex. The NH or OH groups of the residues which produce these bands are likely to be close to the $Q_A$ cofactor, since the positive and negative peaks exhibit relatively large amplitudes in the $Q_A^-/Q_A$ spectrum and quite distinct features are observed over this spectral region in $Q_B^-/Q_B$ spectra [19]. The peaks (at both hydration levels) at 3516 (negative) and 3504 (positive) $cm^{-1}$ are absent in the $Q_A^-/Q_A$ spectrum. The 3504 $cm^{-1}$ band shows a peculiar behavior, strongly increasing its amplitude (relative to other bands) in the $r=11\%$ $P^+Q_A^-/PQ_A$ difference spectrum.

The broad positive band peaking at 3417 $cm^{-1}$ in the $H_2O$ hydrated film equilibrated with $H_2O$ at $r=76\%$ is replaced by a positive 3425 $cm^{-1}$ band in the $D_2O$ hydrated sample. No shift of this band is observed after $H_2^{18}O$ substitution. The same behavior characterizes the peaks at 3386 (positive), 3375 (negative) and 3352 (negative) $cm^{-1}$ in the $H_2O$ hydrated film equilibrated with $H_2O$ at $r=76\%$ which also do not seem to change upon $H_2^{18}O/H_2O$ exchange, and apparently undergo a small upshift of 2–4 $cm^{-1}$ upon $D_2O$ replacement. These observations preclude the assignment of the above bands to hydrogen bonded water molecules. We tentatively attribute the bands to NH or OH groups of the protein which do not undergo H/D exchange, but which apparently experience in $D_2O$ a slight weakening of the strength of the hydrogen bonds in which they are implicated.

The comparison between the "hydrated" ($r=76\%$) and "dehydrated" ($r=11\%$) RC-LDAO films (Fig. 4B) indicates, besides the shift of the 3498 and 3483 $cm^{-1}$ bands described above, additional significant alterations in the light-induced FTIR difference spectrum. Shifts (2–6 $cm^{-1}$) to higher wavenumbers can be noticed for the three peaks at 3386, 3361, and 3344 $cm^{-1}$. Much more prominent differences can be observed between 3300 and 3240 $cm^{-1}$. Over this spectral interval, while in the $H_2O$ hydrated film equilibrated with $H_2O$ at $r=76\%$ negative peaks appear at 3305, 3288 and 3240 $cm^{-1}$ and positive ones at 3302 and 3269 $cm^{-1}$, in the dehydrated films (equilibrated with $H_2O$ at $r=11\%$ ) a large positive peak is detected at 3275 $cm^{-1}$, delimited by two negative peaks at 3300 and 3255 $cm^{-1}$. As no significant shift is observed upon $D_2O$ and $H_2^{18}O$ substitution (see above), these bands should originate from NH or OH stretching modes of RC amino acid residues which are not exposed to $D_2O/H_2O$ exchange and which respond differently to photoexcitation in the hydrated ($r=76\%$) and in the dehydrated ($r=11\%$) RC–detergent complex. The latter observations point to the involvement of amino acid residues of the RC in conformational changes induced by a prolonged (20 s) illumination of the RC. These conformational dynamics appear to be affected by the hydration level of the RC–detergent complex, thus suggesting a tight dynamical coupling between the protein and its hydration shell (see Discussion). Since, as outlined in Section 1, the kinetics of recombination of the light-induced $P^+Q_A^-$ state is a sensitive probe of the conformational relaxation induced by charge separation, we report in the following on the kinetics of this ET process, analyzed under the same photoexcitation and hydration conditions employed in the FTIR measurements described above.

### 3.5. $P^+Q_A^-$ recombination kinetics in hydrated and dehydrated RC-LDAO films

Fig. 5A compares the absorbance changes at 422 nm induced by a 20 s photoexcitation in RC-LDAO films equilibrated with water vapour at relative humidity $r=76\%$ (trace *a*) and $r=11\%$ (trace *b*). Absorbance changes at this wavelength are due to the redox changes of the $P^+/P$ couple, and, to a minor extent, to those of $Q^-/Q$ [35]. The signals monitor therefore the kinetics of $P^+Q_A^-$ formation upon photoexcitation and recombination in the dark. Although the RC concentration and the optical path of the two films are comparable (as evaluated from the absorbance of the bacteriochlorin bands between 650 and

[2] To our knowledge, only complete 4000-1000 $cm^{-1}$ spectra have been reported so far for *Rb. spaheroides* RCs [39,40,54,55], where the attention was always focused on the electronic $P^+$ band.

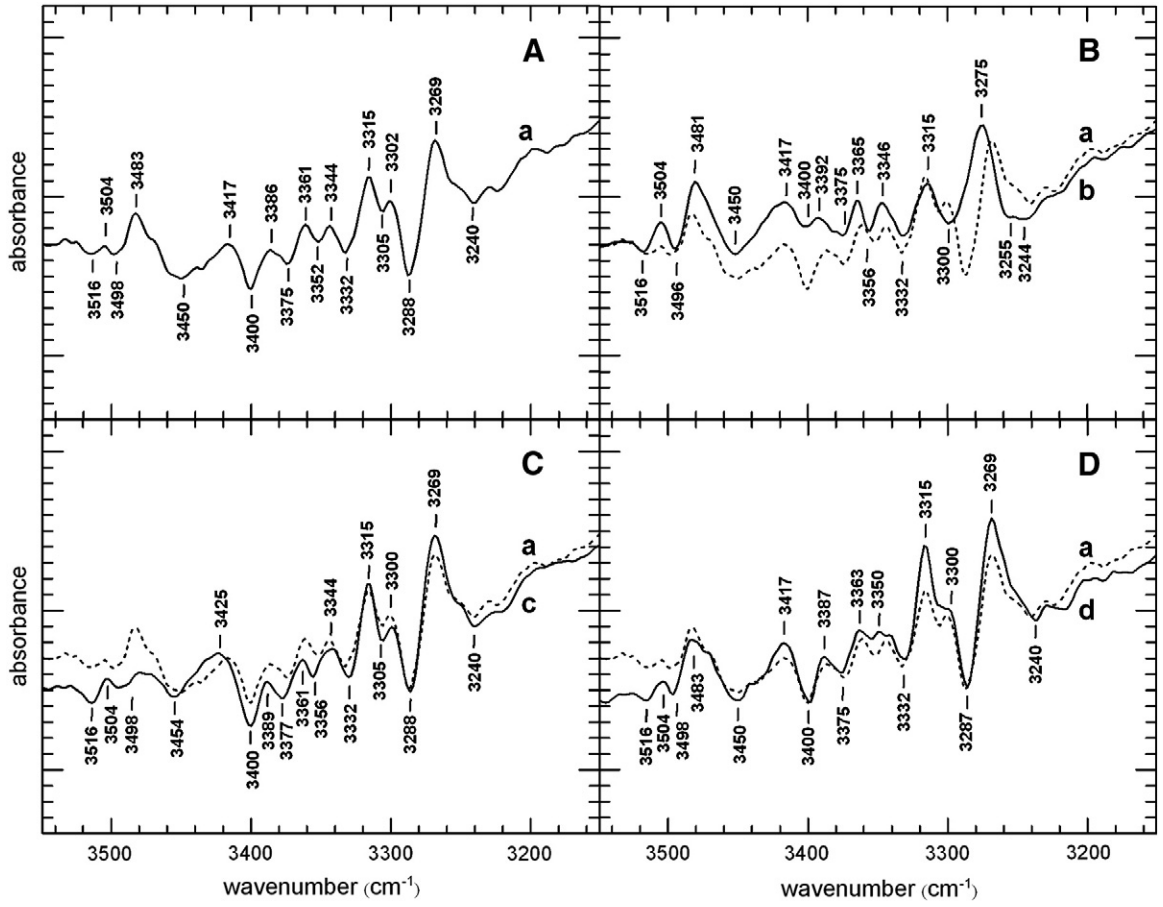


**Fig. 4.** Light induced $P^+Q_A^-/PQ_A$ FTIR difference spectra measured in the 3550–3150 $cm^{-1}$ range on RC-LDAO films hydrated with $H_2O$ at a relative humidity $r = 76\%$ (panel A, continuous curve *a*) and $r = 11\%$ (panel B, continuous curve *b*). The corresponding spectra recorded at $r = 76\%$ after $D_2O$ or $H_2^{18}O$ substitution are shown in panel C (continuous curve *c*) and panel D (continuous curve *d*), respectively. For the sake of comparison the spectrum in panel A, measured at $r = 76\%$ in $H_2O$, is also shown in panel B, C, and D with a dashed line. The increment between major ticks of the y-axis corresponds to $1 \times 10^{-5}$ absorbance units.

900 nm) the amplitude of the light-induced signal is more than three times larger in the "hydrated" sample ($r = 76\%$) as compared to the "dehydrated" sample ($r = 11\%$), indicating that the fraction of the RC population which upon continuous illumination is maintained steadily in the $P^+Q_A^-$ charge separated state is much lower in the dehydrated conditions. The relative levels of $P^+Q_A^-$ optically detected in the "hydrated" and "dehydrated" films agree well with those estimated from the FTIR electronic band of $P^+$ around 2600 $cm^{-1}$ (see Section 3.1). Such a behavior is qualitatively consistent with the observation that, following a laser pulse, the $P^+Q_A^-$ recombination process is strongly accelerated in dehydrated RC–detergent complexes [20]. Since the steady level of charge separation under continuous photoexcitation is determined by the kinetic competition between the processes of charge separation and recombination [56], an acceleration of the latter is expected to result in a decreased steady $P^+Q_A^-$ signal observable in continuous light.

From Fig. 5A it also appears that, following a 20 s period of continuous illumination, the overall decay of the light-induced $P^+Q_A^-$ signal is dramatically slower in the hydrated RC film ($r = 76\%$) as compared to the dehydrated one ($r = 11\%$). In the latter sample the decay is unresolved over the time scale of seconds, while, at $r = 76\%$, about 10% of the photoxidized RCs are still in the charge separated state after a dark time of about 12 s. The charge separated state is therefore strongly stabilized in the hydrated RC-LDAO film as compared to the dehydrated sample. A closer inspection of the decay kinetics (which are shown expanded in time and normalized to the maximal amplitude in Fig. 5B) evidences that in the dehydrated sample the decay is essentially monophasic, with an half time of approximately 80 ms; at variance in the hydrated condition ($r = 76\%$) the recombination process is distinctly biphasic, including a fast phase comparable in rate to the decay observed in the dehydrated sample ($r = 11\%$) and an extremely slow kinetic phase, that accounts for at least half of the total amplitude, characterized by an half time of approximately 5 s.

Fig. 5C compares, over a logarithmic time scale, the kinetics shown in panels A and B, recorded after prolonged, continuous illuminations, with $P^+Q_A^-$ recombination kinetics measured in the same RC films following a short (7 ns) laser pulse. As previously reported [20], $P^+Q_A^-$ recombination after a laser pulse is significantly accelerated upon dehydration of the RC–detergent film. Furthermore, the non exponential character of the decay becomes more evident in the dehydrated film (at $r = 11\%$), which exhibits a recombination kinetics strongly distributed in rate (see Fig. 5C). As a consequence a quantitative description of the kinetics, $N(t)$, requires a continuous distribution $p(k)$ of rate constants, according to:

$$N(t) = \frac{P^+Q_A^-(t)}{P^+Q_A^-(0)} = \int_0^\infty p(k)e^{-kt}\mathrm{d}k \tag{1}$$

We found that the Gamma distribution provides an adequate expression for $p(k)$ [20], so that, in the time domain, $N(t)$ can be simply fitted to a power law of the form:

$$N(t) = (1 + k_0 \cdot t)^{-n} \tag{2}$$

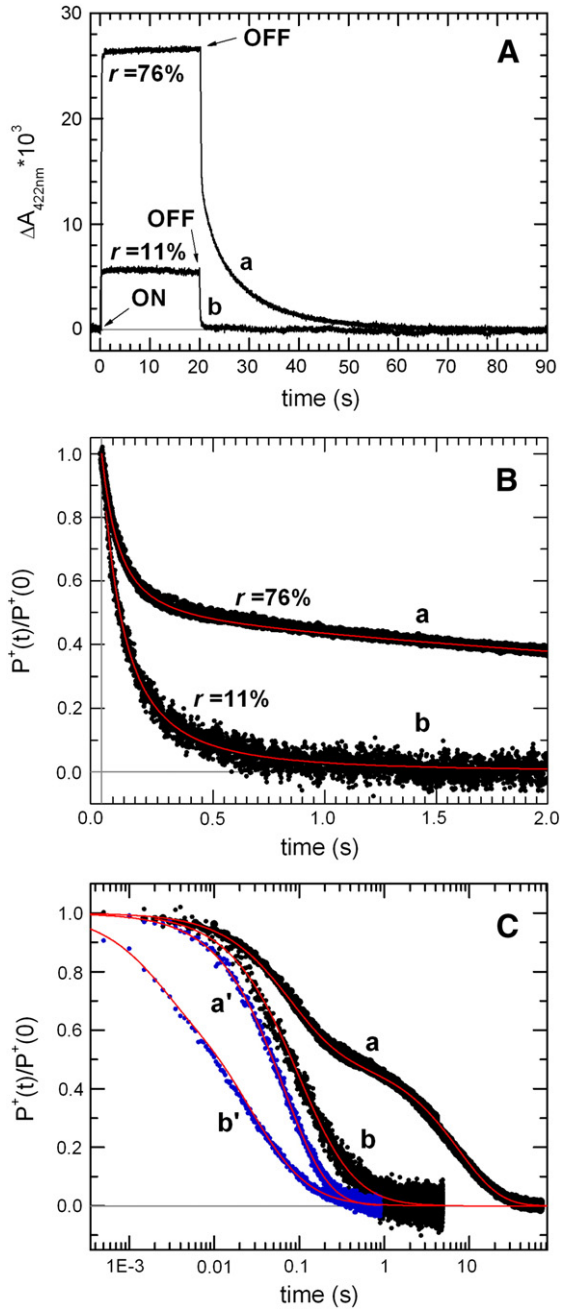


**Fig. 5.** $P^+Q_A^-$ charge recombination kinetics in RC-LDAO films characterized by two different hydration levels, i.e. equilibrated with water vapour at relative humidity $r=$ 76% and $r=11\%$. Panel A. Time course of the absorbance change induced at 422 nm by a 20 s period of continuous illumination in the hydrated ($r=76\%$, trace *a*) and dehydrated ($r=11\%$, trace *b*) RC film. Panel B. Normalized kinetics of $P^+Q_A^-$ recombination obtained from the traces shown in panel A by expanding the time scale. In the case of the decay recorded at $r=76\%$, which encompasses 5 orders of magnitude in time, data have been acquired in two sets with different time resolutions (0.5 ms and 10 ms per point ). Both data sets were used simultaneously when fitting the kinetics. The red curves represent best fit to a single power law (Eqs. (2) and (3)) for the decay at $r=11\%$, and to the sum of two power laws (Eq. (4)) for the decay at $r=76\%$. Values of the best fitting parameters are: at $r=76\%$ , $A_F=0.51$, $\langle k_F\rangle=13.0\ s^{-1}$, $\sigma_F=8.4\ s^{-1}$, $\langle k_S\rangle=0.13\ s^{-1}$, $\sigma_S=4.9\cdot10^{-2}\ s^{-1}$; at $r=11\%$ , $\langle k\rangle=10.2\ s^{-1}$, $\sigma=7.4\ s^{-1}$. Panel C. The normalized kinetics of $P^+Q_A^-$ recombination after a 20 s period of continuous photoexcitation (from panels A and B) are compared with the kinetics recorded after a 7 ns laser pulse in RC-LDAO films equilibrated at $r=76\%$ (blue trace *a'*) and $r=11\%$ (blue trace b'). Best fit to a single power law of the decays after the laser pulse are plotted in red and correspond to the following values of parameters: at $r=$ 76%, $\langle k\rangle=15.2\ s^{-1}$, $\sigma=7.0\ s^{-1}$; at *r* 11%, $\langle k\rangle=38.8\ s^{-1}$, $\sigma=29.8\ s^{-1}$.

where the fitting parameters $k_0$ and $n$ are related to the average rate constant $\langle k\rangle$ and to the width $\sigma$ of the Gamma rate distribution by:

$$k_0=\frac{\sigma^2}{\langle k\rangle}\ ,\ n=\frac{\langle k\rangle^2}{\sigma^2} \tag{3}$$

In full agreement with our previous results [20], fitting the decays of $P^+Q_A^-$ induced by a laser pulse to Eq. (2) (see Fig. 5C) shows that the average rate constant increases upon dehydration from $\langle k\rangle=$ 15.2 $s^{-1}$ (at $r=76\%$) to $\langle k\rangle=38.8\ s^{-1}$ (at $r=11\%$); the width of the rate distribution also increases in parallel from $\sigma=7.0\ s^{-1}$ (at $r=76\%$) to $\sigma=29.8\ s^{-1}$ (at $r=11\%$). As summarized in the Introduction, these effects, which mimic at room temperature similar effects observed in water–glycerol RC systems frozen in the dark at cryogenic temperatures [4,7], have been taken to reflect a strong inhibition of the RC relaxation from the dark- to the light-adapted conformation as well as a dramatic hindering of the interconversion between RC conformational substates [7,21,24].

Somewhat surprisingly, the kinetics of $P^+Q_A^-$ recombination following prolonged, continuous illumination, differ substantially at both hydration levels from the corresponding ones measured after a short laser photoexcitation. The kinetics measured after continuous illumination in the RC film equilibrated at $r=11\%$ fit a single power law, with $\langle k\rangle=10.2\ s^{-1}$ and $\sigma=7.4\ s^{-1}$. It appears that the kinetic effects of dehydration observable after a laser flash have totally reverted following a long continuous photoexcitation, since the kinetics is even slower than the one recorded after a laser pulse in the hydrated film, at $r=76\%$ (see Fig. 5C). The kinetics observed after continuous light in the dehydrated film are comparable to those observed in a RC solution at room temperature [4]. In the hydrated film ($r=76\%$) the recombination kinetics after a 20 s continuous photoexcitation is also markedly slowed down as compared to the one recorded at the same hydration after a laser pulse (see Fig. 5C). In view of their biphasic and distributed character, the recombination kinetics recorded after 20 s photoexcitation at $r=76\%$ have been fitted to the sum of two power laws, i.e. to:

$$N(t)=A_F\left(1+\frac{{\sigma_F}^2}{\langle k_F\rangle}\cdot t\right)^{-\left(\frac{\langle k_F\rangle}{\sigma_F}\right)^2}+(1-A_F)\left(1+\frac{{\sigma_S}^2}{\langle k_S\rangle}\cdot t\right)^{-\left(\frac{\langle k_S\rangle}{\sigma_S}\right)^2} \tag{4}$$

where $A_F$ is the relative amplitude of the fast kinetic phase, $\langle k_F\rangle$, $\langle k_S\rangle$ and $\sigma_F$, $\sigma_S$ are the average rate constant and distribution width of the faster and slower kinetics phases, respectively. The values of the obtained kinetic parameters (reported in the caption of Fig. 5) show that the kinetics of the fast component, accounting for about 50% of the total decay, are comparable to those of the monophasic recombination measured at $r=11\%$ after 20 s photoexcitation. This result suggests that continuous illumination totally removes over the time scale of charge recombination the hindering of the RC conformational dynamics caused by dehydration, as probed by the $P^+Q_A^-$ decay kinetics after a short photoexcitation [57]. In addition, the appearance of a very slow kinetic phase in the hydrated ($r=76\%$) RC-LDAO films ($\langle k_S\rangle=0.13\ s^{-1}$ and $\sigma_S=4.9\cdot10^{-2}\ s^{-1}$) indicates that in a significant fraction (about 50%) of the RC population the primary charge separated state has undergone a dramatic stabilization as a result of the prolonged (20 s) photoexcitation.

In order to safely attribute the slow phase of the decay measured at 422 nm to a genuine $P^+Q_A^-$ recombination, excluding for instance side reactions which could re-oxidize the photo-reduced $Q_A^-$, leaving the photoxidized $P^+$ without a recombination partner, we have measured in parallel the kinetics of absorbance changes at 450 nm. At 422 nm the contribution of $P^+$ dominates the absorption change, with the spectral contribution of the semiquinone accounting for about 14% of the total. At 450 nm, at variance, the relative contribution of $Q^-$ increases to about 40% at the expense of that due to $P^+$ [20]. Since essentially the same decay kinetics have been observed at the two

wavelengths under all the hydration and photoexcitation conditions tested (data not shown), we attribute also the extremely slow kinetic component observed in the hydrated RC film after a 20 s photoexcitation to $P^+Q_A^-$ recombination. We can also exclude that the slow phase of the decay measured at 422 and 450 nm reflects recombination from the secondary quinone acceptor $Q_B^-$, possibly due to the inability of o-phenantroline to fully inhibit $Q_A^-$ to $Q_B$ ET under continuous bright illumination lasting for 20 s. In fact, the difference FTIR light-minus-dark spectrum recorded under the same illumination regime shows in the 1670–1600 $cm^{-1}$ and in the 3750–3550 $cm^{-1}$ regions the typical features of $Q_A^-/Q_A$, quite distinct from those of $Q_B^-/Q_B$ (see Sections 3.2 and 3.3).

We recall that comparably slow kinetic phases of $P^+Q_A^-$ recombination have been observed in RC solution samples at room temperature following bright continuous photoexcitation [8,11,15], exhibiting a complex dependence upon the duration and the intensity of illumination. Such a dramatic stabilization of the primary charge separated state, which resembles the one observed by us in the hydrated RC film, has been proposed to originate from slow structural changes of the RC protein occurring during continuous illumination [8,11,15].

In conclusion the kinetic analysis of $P^+Q_A^-$ recombination after prolonged continuous photoexcitation suggests a correlation between the light-induced FTIR spectral changes sensitive to the hydration state (Sections 3.2–3.4) and the strong stabilization of the primary charge separated state which is observed after continuous illumination only in the hydrated RC film.

## 4. Discussion

The roles of water molecules, belonging to the protein hydration shell, to the bulk solvent or placed inside the protein, in governing specific dynamics and functions of proteins are receiving a growing interest [58–60]. Several FTIR difference studies have contributed to clarify the function of internal water molecules in different membrane proteins, as bacteriorhodopsin [46], cytochrome c oxidase [48], photosystem II [47], and the bacterial RC [19,28]. Recently, light-induced $Q_A^-/Q_A$ FTIR difference spectra in the 3700–3450 $cm^{-1}$ range have been measured in hydrated RC films equilibrated at $r = 98\%$ [19] and vibrational changes attributed to weakly hydrogen bonded water molecules have been observed as a consequence of $Q_A^-$ formation; on this basis, the reorientation of water dipoles near $Q_A$ has been tentatively proposed as a conformational (dielectric) relaxation stabilizing the $P^+Q_A^-$ state in the RC.

We have extended the study by Iwata and co-workers [19] in several directions: i) we examined $P^+Q_A^-/PQ_A$ FTIR difference spectra rather than $Q_A^-/Q_A$ spectra, in order to detect events which are induced by the electric field due to the radical pair ($P^+Q_A^-$), thus including processes which might be coupled to the formation of $P^+$; ii) two hydration states of the RC film have been considered to study the effect of the protein hydration level on the water differential bands observed by Iwata et al. [19]; iii) a large IR spectral range has been explored to reveal the possible contribution of other chemical groups to the stabilization of the charge separated state $P^+Q_A^-$; iv) we have analyzed the charge recombination kinetics of the $P^+Q_A^-$ state following the same illumination regime used in the FTIR measurements, in order to probe the stability of the charge separated state and correlate it with the light-induced IR spectral changes observed in parallel. We aimed at linking the information on RC dynamics and relaxation provided by charge recombination kinetics to the structural information emerging from FTIR light-minus-dark difference measurements. At the two hydration levels studied, in fact, the kinetics of $P^+Q_A^-$ recombination after the 20 s continuous illumination period used to record FTIR difference spectra, were markedly different, indicating a more stabilized charge separation in the hydrated RC-LDAO film as compared to the dehydrated one (Fig. 5).

Three differential bands have been resolved in the $P^+Q_A^-/PQ_A$ FTIR difference spectrum of hydrated RC-LDAO films in the 3750–3550 $cm^{-1}$ range (Fig. 2), which, in view of the effects of water substitution with $D_2O$ and $H_2^{18}O$, have been attributed to weakly hydrogen bonded water molecules. From the high efficiency of isotope substitution, it can be concluded that they are located in sites accessible to the solvent. Among these three bands, the positive one at 3664 $cm^{-1}$ and the negative one at 3587 $cm^{-1}$ peak at the same wavenumbers of two of the difference bands detected by Iwata et al. [19] in $Q_A^-/Q_A$ spectra. We identify therefore these bands with the ones observed by Iwata and coworkers in the presence of the only semiquinone anion $Q_A^-$, attributed to weakly hydrogen bonded water molecules in the vicinity of $Q_A$, which respond with a vibrational rearrangement to its photoreduction. In the crystallographic structure determined by Koepke and co-workers [61], refined up to 1.87 Å (PDB: 2J8C), a cluster formed by 15 water molecules is located in a region within 10 Å from $Q_A$. A large part of these molecules has been also identified in previous structures at lower resolution [62,63], including in particular the closest water molecules, sitting at ~5 Å from $Q_A$ (WH2003, WM2111, WM2113). Since the network of water molecules approaches the cytoplasmic surface of the RC, it is likely that many of the water sites which constitute the cluster are accessible to the bulk solvent. Some water molecules from this cluster, not strongly H bonded, might in principle be responsible for the bands detected at 3664 $cm^{-1}$ and 3587 $cm^{-1}$ in the difference spectrum. The average value of the B factors determined for the water molecules which form the cluster is $(28.0 \pm 14.7)$ $Å^2$, suggesting a non-negligible thermal motion and/or disorder. At least some of these water molecules might be sufficiently mobile to take part in the dielectric relaxation which follows the primary charge separation. Interestingly the positions of most of the water molecules located within 10 Å from $Q_A$ in the RC structure of wild type *Rb. sphaeroides* [61], appear to be conserved in the RC structure of *Rb. sphaeroides* R26 [64] (PDB: 2GNU), as well as in *Blastochloris viridis* [65] (PDB: 2WJN).

In the spectral interval between the two bands at 3664 $cm^{-1}$ and 3587 $cm^{-1}$, our $P^+Q_A^-/PQ_A$ difference spectrum is somewhat different from the $Q_A^-/Q_A$ spectrum reported by Iwata and colleagues. They have identified a small negative bands at 3622 $cm^{-1}$, also attributed to weakly hydrogen bonded water molecules in the vicinity of $Q_A^-$; at variance we have observed a rather broad, more intense positive band at approximately 3628 $cm^{-1}$, which undergoes the expected 10 $cm^{-1}$ shift upon water substitution with $H_2^{18}O$, thus indicating that it originates from water molecules. Although we cannot exclude that these differences are due, at least in part, to some spectral interference from other molecules, it is likely that the broad band detected by us at 3628 $cm^{-1}$ originates from water molecules interacting predominantly with the primary donor P, rather than with the $Q_A$ acceptor. The potential role of water molecules near P in light-induced long-lived conformational changes has been recently discussed by Deshmukh and coworkers [12]. Based on the crystallographic structure determined by Ermler et al. [62] (PDB: 1PCR), these authors identified in particular 5 water molecules (WL728, WL729, WL723, WL736, WL737) located in key positions to participate potentially in dielectric relaxation. The positions of these water molecules are conserved in the better resolved structure determined by Koepke et al. [61] (corresponding to WL2113, WL2075, WL2058, WM2092, WL2072, respectively (PDB: 2J8C)), which shows within 10 Å from the P dimer 27 water molecules, as compared to 10 water molecules identified within the same distance by Ermler et al. [62]. The average B factor for the cluster of the 27 water molecules located in the region within 10 Å from P (see PDB: 2J8C) is $(41.0 \pm 9.1)$ $Å^2$, with values ranging between 19 $Å^2$ and 69 $Å^2$ for the 5 water molecules closest to the dimer. Several water molecules belonging to this cluster might therefore be sufficiently mobile to reorient in the electric field generated by the light-induced charge separation, thus contributing significantly to stabilize the $P^+Q_A^-$ state by dielectric screening. As for the water molecules identified in the $Q_A$ region, the positions of

many water molecules located within 10 Å from the P dimer in the wt *Rb. sphaeroides* structure [61] are conserved in the structures of the RC from *Rb. spaheroides* R26 [64] (PDB: 2GNU), and from *Blastochloris viridis* [65] (PDB: 2WJN). We notice however that the water molecules sensed by the light-minus-dark difference FTIR spectrum in the 3750–3550 $cm^{-1}$ region in response to $P^+Q_A^-$, being weakly hydrogen bonded, may be too disordered and thus not observable in X-ray crystal structures.

When the water content of hydrated RC films (equilibrated at $r=$ 76%) was markedly reduced (at $r=11\%$) we have observed a drastic reduction in the amplitude (or possibly the disappearance) of the three differential bands discussed above. The simplest interpretation is that the large majority of the weakly bound water molecules which originate the light-induced bands have been removed upon dehydration. However, since the depletion of the RC hydration shell obtained at $r=11\%$ causes a strong inhibition of the RC/solvent dynamics [20], it is also possible that the water molecules responsible for the bands at 3664, 3628 and 3587 $cm^{-1}$ at $r=76\%$ are still present, but sufficiently "immobilized" to be unable to reorient and/or undergo vibrational rearrangements in response to light-induced charge separation. Also in this case a substantial decrease of the difference bands is expected.

The strong reduction of the difference water bands observed upon dehydration occurs in parallel with a strong destabilization of the primary charge separated state, as inferred from the $P^+Q_A^-$ recombination kinetics recorded after a 20 s period of continuous photoexcitation (Fig. 5), i.e. following the same illumination regime under which the difference FTIR spectra have been acquired. This suggests a correlation between the dynamics of the weakly hydrogen bonded water molecules contributing to the difference bands in the 3750–3550 $cm^{-1}$ range and the conformational processes which stabilize the $P^+Q_A^-$ state.

Our results indicate that the stabilization of the charge separated state depends not only upon the hydration level of the RC complex, but also upon the duration of the photoexcitation period. Fig. 5 shows that, at both hydration levels, a prolonged (20 s) illumination, as compared to a 7 ns (laser) photoexcitation, leads to a considerably more stable $P^+Q_A^-$ state (i.e. to a substantial overall slowing of $P^+Q_A^-$ recombination). The effects observed in the RC film equilibrated at $r=11\%$ indicate that the inhibition of the RC relaxation probed by the charge recombination kinetics after the laser flash is essentially reverted following a prolonged illumination. In the hydrated RC film, however, an additional relaxation process appears to take place upon continuous illumination. At $r=76\%$, in fact, the continuous 20 s photoexcitation results in a clearly biphasic decay of the charge separated state. The fast distributed phase exhibits kinetic parameters similar to those observed in solution, again showing a reversion of the inhibition of the RC dynamics observed after a laser flash. The second kinetic phase, accounting for half of the kinetics, decays in the tens-of-seconds time scale, with an average rate constant ($<k_S>=0.13\ s^{-1}$) smaller by two orders of magnitude. The presence of this slow recombination phase strongly suggests that different, additional relaxation processes have occurred during the prolonged illumination at this hydration level: these processes increase strongly the stability of the primary charge separated state in about half of the RC population. Extremely slow components in the $P^+Q_A^-$ decay, comparable in rate to the one described above, have been observed in RC solution samples at room temperature as a consequence of continuous illumination for time periods ranging from seconds to minutes, the effective charge recombination time distributions depending on the duration and intensity of photoexcitation [8,11]. This extra-stabilization of the charge separated state has been attributed to additional conformational changes, occurring over a much longer time scale [8,9,11,14,15], as compared to the relaxation from the dark- to the light-adapted conformation probed by the $P^+Q_A^-$ recombination kinetics recorded after a single laser pulse [4,7]. We propose therefore that, following a long (20 s) photoexcitation, a similar, additional conformational relaxation, occurs in hydrated ($r=76\%$) RC films, which is inhibited by dehydration in RC films equilibrated at $r=11\%$.

To summarize, it appears that, following long periods of continuous photoexcitations, the difference in the RC dynamics between the "hydrated" ($r=76\%$) and the "dehydrated" ($r=11\%$) system concerns mainly the slow conformational relaxation leading to extra-stabilization of the charge separated state: this process takes place in the "hydrated" system, but is absent (inhibited) in the "dehydrated" RC. The light-induced difference bands in the 3750–3550 $cm^{-1}$ range (Fig. 2), which are strongly reduced upon dehydration, are observed during such a long photoexcitation of the system. We suggest therefore that the spectral response of the weakly bound water molecules which originate these bands is more likely related to the slow (lifetime $\tau\sim10$ s), extra-stabilizing RC conformational relaxation, rather than to the fast ($\tau<10^{-2}$ s) conformational change evidenced by laser-flash experiments.

Besides the difference bands ascribed to water molecules weakly bound to the RC, other features of the light-minus-dark difference spectrum are affected by the hydration level of the RC, including, in the 1750–1450 $cm^{-1}$ region (Fig. 3), spectral contributions attributed to modes of the special pair P, and several bands in the 3550–3150 $cm^{-1}$ range (Fig. 4). In this spectral region, which, to our knowledge, has not been analyzed before in *Rb. sphaeroides* RCs, no band could be attributed to water molecules, since we have not observed any shift upon isotopic substitution with $D_2O$ and $H_2^{18}O$. Several changes were observed as a consequence of water depletion: shifts of a few $cm^{-1}$ for bands between 3500 and 3300 $cm^{-1}$, and strong alterations in the spectral features between 3300 and 3240 $cm^{-1}$. Although no attribution to specific amino acidic residues is possible for these differential bands, we can conclude that several OH or NH groups of amino acidic chains undergo relevant structural changes in parallel with dehydration, and, therefore, in parallel with the inhibition of the RC conformational relaxation, as inferred from the kinetic analysis of $P^+Q_A^-$ recombination (Fig. 5). Some of these OH or NH groups (e.g. those responsible for the bands at 3498 and 3483 $cm^{-1}$ in the hydrated film) are most likely located on the cytoplasmic side of the RC (see Section 3.4 and [19]). This observation is in line with X-ray diffraction data collected at low temperature in *Rb. sphaeroides* RC crystals during continuous illumination with bright light [9]. The comparison with data acquired in crystals that were not illuminated showed in fact significant movements (up to 0.7 Å) in a large number of amino acidic residues of the H subunit between ProH121 and ThrH226.

As mentioned in the Results section, upon dehydration we observed a shift in the 9-keto C=O band of the $P_M$ bacteriochlorophyll of the oxidized primary donor (1701 $cm^{-1}$ in $r=76\%$ spectrum, 1703 $cm^{-1}$ in $r=11\%$ spectrum). This shift strongly suggests a change in the interaction between the 9-keto C=O of $P_M$ (at least when P is oxidized) and the surrounding environment; this change could be in the strength of the hydrogen-bonding state of the 9-keto C=O and/or in the dielectric constant of its immediate environment. In agreement with this observation, slight spectral modifications in passing from $r=76\%$ to $r=11\%$ are visible in all the regions where $P^+$ bands lie (~1550, ~1480, ~1300 $cm^{-1}$). This demonstrates that also the periplasmic side is affected by the dehydration level of the RC, in agreement with the tentative assignment of the 3628 $cm^{-1}$ band to a weakly hydrogen-bonded water molecule close to P. Therefore, not only amino acidic residues and bound water molecules localized on the cytoplasmic acceptor side of the RC might be involved in the light-induced conformational relaxation, but also amino acidic residues and/or water molecules localized in the opposite periplasmic donor side. This notion is consistent with recent X-ray diffraction and functional studies aimed to elucidate the structural basis of RC conformational changes induced by photoexcitation. By applying time-resolved Laue diffraction to catch light-induced structural changes in the RC of *Blastochloris viridis*, Wöhri and coworkers [10] found that the side chain of TyrL162, a residue strictly

conserved in purple bacterial RCs which lies next to P special pair, moves 1.3 Å closer to P after photoactivation. Furthermore, by studying the effect of prolonged illumination (1–2 min) on $P^+Q_A^-$ kinetics in 11 mutants of *Rb. sphaeroides* RC characterized by different hydrogen bonding patterns of the primary electron donor P, it was concluded that the relaxation event which stabilizes the $P^+Q_A^-$ state in the second time scale could be the deprotonation of one or more amino acid residues in the vicinity of the special pair P [13]. Interestingly, it has been proposed that the pathway of the released protons to the solvent involves not only amino acid side chains, but mainly the structural water molecules in the vicinity of P [12,13]. Finally the small spectral changes in both the amide I and amide II regions in passing from $r=$ 76% to $r=11\%$ suggest that also the response of the protein backbone to the charge separation is sensitive to the dehydration level.

## 5. Conclusions and outlook

As a whole our results indicate that a partial depletion of the hydration shell of the RC affects the structural/dynamical response to light-induced charge separation of several groups, belonging to the primary donor P and to amino acidic residues, both on the acceptor and donor side of the RC, as well as water molecules weakly bound to the protein. This finding is fully consistent with the notion that the probed structural/dynamical rearrangements induced by the primary charge separated state of the RC are tightly coupled to the dynamics of the hydration shell of the complex [20,25], representing therefore dielectric β relaxations [59]. It is tempting to propose that all the pigment–protein groups considered above, as well as the weakly bound water molecules which give rise to the FTIR responses affected by the hydration level of the RC, contribute, possibly over different time-scales and to a different extent, to stabilize the $P^+Q_A^-$ charge separated state in a collective process. Our results, obtained under prolonged, continuous illumination, do not allow, however, to disentangle conformational changes which primarily stabilize by dielectric screening the $P^+Q_A^-$ state from structural (vibrational) alterations which occur at a later stage or are a consequence of the primary conformational changes. Time-resolved FTIR difference measurements, aimed to better clarify the nature and the sequence of the relaxation processes involved in short- and long-lived conformational changes, are underway in our laboratories.

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